Unesterified fatty acid metabolism in rat brain.

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The reader's attention is drawn to the fact that since this thesis was submitted the work of Gullis and Rowe (1975 a,b & c) has been withdrawn. Please see the following references:-

1. R.J. Gullis (1977) Nature (London) 265, 764

2. C.E. Rowe (1977) Biochem. J. 164, 287-288

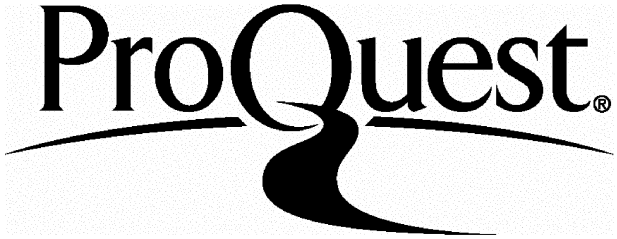
UNESTERIFIED FATTY ACID METABOLISM IN RAT BRAIN

submitted by Robert W. Bonser

for the degree of Ph.D. of the

University of Bath, 1976

R. W. Bonser



TO MUM AND DAD

ACKNOWLEDGEMENTS

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R.W. Bonser

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CONTENTS

SUMMARY

1. The incorporation of [1 -^{14}C] acetate *in vivo* into the three major classes of fatty acid (i.e. fatty acids from phospholipids, neutral glyceride fatty acids and unesterified fatty acids) in rat cerebral cortex has been measured. These measurements have shown that endogenous unesterified fatty acids turn over rapidly *in vivo*, with a half-life of approximately five minutes. Furthermore, under normal non-stimulated conditions unesterified fatty acids are rapidly incorporated into phospholipids and neutral glycerides.

2. A washing procedure has been developed which effectively reduces the contamination of the fatty acid extracts by unmetabolized acetate present in the excised tissue.

3. It has been shown that radioactively labelled acetate is rapidly incorporated into the unesterified fatty acids associated with subcellular fractions of rat cerebral cortex *in vivo*.

4. The level of long-chain fatty acyl-CoA esters in rat brain has been determined. The incorporation of [1 -^{14}C] acetate *in vivo* into the long-chain fatty acyl-CoA esters of rat brain has also been measured. These measurements have shown that the thioesters turn over rapidly *in vivo* with a half-life of approximately thirty seconds.

5. The subcellular localization of the enzyme long-chain fatty acyl-CoA hydrolase has been investigated. The results have shown that the enzyme is almost entirely soluble in nature and that it is not localized in any particular subcellular fraction from rat cerebral cortex.

6. A possible control mechanism for the enzyme *in vivo* has also been investigated. The enzyme is inhibited by Ca^{2+} and Mg^{2+} and by bovine serum albumin, but is not affected by neurotransmitters and free fatty acids.

7. The role of the enzyme long-chain fatty acyl-CoA hydrolase in brain has been examined and it is suggested that the enzyme may be involved in the maintenance of the unesterified fatty acid pool in brain.

INTRODUCTION

INTRODUCTION

The first detailed report of unesterified fatty acid in brain was made by Rowe in 1964. Rowe used four different methods to isolate unesterified fatty acids from mouse brain. The distribution and yields of the acids were similar whether they were isolated by chromatography on silica gel, partition between solvents or, as their methyl esters, by thin-layer chromatography on alumina. The principle acids isolated were palmitic, stearic, oleic and arachidonic acids. The results indicated that mouse brain contained appreciable quantities of unesterified fatty acids and that they were unlikely to be an artifact arising from the breakdown of esterified fatty acid during the isolation procedure. Since the work of Rowe several groups of workers have confirmed the presence of unesterified fatty acids in brain (Lunt and Rowe, 1968, 1971; Bazan and Joel, 1968; Bazan, 1971a; Cenedella, Galli and Paoletti, 1975; Lunt and James, 1976). Unesterified fatty acids have been found distributed among different gross neuroanatomical regions of the brain (Bazan *et al.*, 1971; Bazan and Joel, 1968; Bazan and Rakowski, 1970) as well as in subcellular particles of neural tissue (Lunt and Rowe, 1968; Price and Rowe, 1972).

The reported concentrations of unesterified fatty acid in the brain differ greatly. Galli and ReCecconi (1967) found amounts

corresponding to 2-3 mg/g whole brain, Lunt and Rowe (1968) found levels equivalent to 150 µg/g and Bazan *et al.* (1971) reported levels of 30-40 µg/g in rat brain excised and frozen or homogenised within thirty seconds of decapitation. Bazan and co-workers suggested that the discrepancy between the various reported levels of the acids could be explained by a rapid increase in brain unesterified fatty acid content, which they were able to show occurs during the first few minutes after decapitation. Bazan further suggested that the rapid increase may be due to normal enzymatic breakdown of esterified fatty acid, rather than tissue autolysis. Recently Cenedella, Galli and Paoletti (1975) have attempted to resolve the controversy concerning the level of rat brain unesterified fatty acid. Cenedella used the focussed microwave irradiation technique described by Guidotti *et al.* (1974), which was originally designed to minimize changes *post mortem* of cyclic nucleotides, DOPA and choline, whilst preserving brain morphology. The level of unesterified fatty acid reported by Cenedella, Galli and Paoletti was 90 µg/g whole brain. This value probably represents the true level *in vivo* of rat brain unesterified fatty acid, since the rapid increase in unesterified fatty acid observed by Bazan *et al.* (1971) following decapitation was absent in rats killed by focussed microwave irradiation.

Very short periods of ischaemia and electroshock treatment produce a great increase in the size of the brain unesterified

fatty acid pool (Bazan, 1970; Bazan and Rakowski, 1970; Bazan, 1971b). Drug-induced convulsions and cobalt-induced epilepsy also increase the size of the unesterified fatty acid pool in brain (Lunt and Rowe, 1971; Bazan, 1971b; Cenedella and Craig, 1973; Lunt and Grove, 1976).

The source(s) of these unesterified fatty acids in the brain is not yet clear. The brain is capable of synthesising fatty acids from acetate *de novo*. Synthesis is accomplished by a soluble cytoplasmic enzyme complex, usually referred to as a fatty acid synthetase system (Volpe and Kishimoto, 1972; Cantrill and Carey, 1975). It is known that during the synthesis of long-chain fatty acids by the fatty acid synthetase system, all of the intermediates are bound to a specific protein, termed an acyl carrier protein. At no stage during the synthesis are free intermediates produced. In addition to the soluble cytoplasmic system biogenesis of fatty acids also occurs in the mitochondria (Aeberhard *et al.*, 1969; Boone and Wakil, 1970). This is essentially a chain elongation process whereby acetyl-CoA is added to long-chain acyl-CoA esters. A third system which occurs in microsomes (Aeberhard *et al.*, 1969; Carey and Parkin, 1975) condenses malonyl-CoA with acyl-CoA derivatives. The rate of fatty acid synthesis by the fatty acid synthetase complex (Cantrill and Carey, 1975) is much too slow to account for the rapid increases in unesterified fatty acid observed during drug-induced convulsions and electroshock treatment.

Furthermore unesterified fatty acids are released from cerebral cortex slices at $0^{o}C$ when lipogenesis from acetate is negligible (Lunt and Rowe,1968).

The mammalian brain is capable of assimilating unesterified fatty acids from the bloodstream. Until quite recently it was believed that the blood-brain barrier restricted the passage of plasma free fatty acids into the central nervous system (Laurell, 1959; Gatt, 1963). However Dhopeshwarkar *et al.* (1969b, 1970, 1971a, b) using radioactive tracer techniques, have shown that palmitic, oleic, linoleic and linolenic acids can be taken up from the bloodstream without prior oxidation to acetate. Furthermore unesterified fatty acid appears to be a preferred form of fatty acid transport into the brain (Dhopeshwarkar *et al.*, 1972).

Unesterified fatty acids may also arise from the hydrolysis of mono, di and triacylglycerols. Brain contains mono, di and triacylglycerol lipases (Myers, 1956; Michell *et al.*, 1973; Vyvoda and Rowe, 1973). The levels of triacylglycerols and diacylglycerols in the brain are small (Rowe, 1969; Sun and Horrocks, 1969; Aveldano and Bazan, 1973; Banschbach and Geison, 1974). Total hydrolysis of acylglycerols by lipase action could not account for the large increases in unesterified fatty acid observed during ischaemia, electroshock treatment and drug-induced convulsions. Recent experiments *in vivo* have shown that

at the onset of ischaemia there occurs in brain a rapid and concomitant production of diacylglycerols and unesterified fatty acids, both surpassing in magnitude the decreases in triacylglycerol levels (Banschbach and Geison, 1974; Aveldano and Bazan, 1975). Hydrolysis of triacylglycerols could only partially contribute to the diacylglycerols and unesterified fatty acids. A decrease in triacylglycerol palmitate and oleate could account for the rise of these fatty acids in the diacylglycerol and unesterified fatty acid pools. However hydrolysis of triacylglycerols cannot account for the appearance of arachidonate and for a large proportion of the stearate and docosahexaenoate in the diacylglycerol and unesterified fatty acid pools.

An alternative source of unesterified fatty acid may be the hydrolysis of long-chain fatty acyl-CoA esters. The presence of the enzyme palmitoyl-CoA hydrolase (EC 3.1.2.2) in brain has been known for some time (Srere *et al.*, 1958; Vignais and Zabin, 1958). The total Coenzyme A content of rat brain is approximately 0.09 μ moles per g of fresh tissue (Kaplan and Lipman, 1948; Jaenicke and Lynen, 1960). Brain also contains a very active palmitoyl-CoA synthetase (EC 6.2.1.3), (Cantrill and Carey, 1975), but to date nothing is known about the rate of turnover of long-chain fatty acyl-CoA esters in the brain. It is unlikely that the hydrolysis of long-chain acyl-CoA esters makes any significant

contribution to the increased levels of unesterified fatty acid seen under conditions of stimulation. However the possibility still exists that under non-stimulated conditions the hydrolysis of long-chain acyl-CoA esters, having a rapid turnover rate, contributes to the maintenance of the unesterified fatty acid pool.

The hydrolysis of phospholipids is the most likely source of the unesterified fatty acids produced under conditions of stimulation. Brain contains high concentrations of phospholipids, which are potential sources of fatty acids. Phospholipase A1 (EC 3.1.1.32), (Gatt *et al.*, 1966; Gatt, 1968), phospholipase A2 (EC 3.1.1.4), (Cooper and Webster, 1970, 1972) and lyso-phospholipase (EC 3.1.1.5), (Leibovitz and Gatt, 1968) are present in brain, and phospholipase A1 and phospholipase A2 have been demonostrated in synaptosomes (Bazan, 1971c; Woelk and Porcellati, 1973; Gullis and Rowe, 1975 a, c). Bazan *et al.* (1971) suggested that noradrenalin may stimulate the hydrolysis of phospholipids in a manner similar to that described for the activation of triglyceride lipase (EC 3.1.1.3) by noradrenalin in brown and white adipose tissue (Correll, 1963; Joel, 1965). Noradrenalin activates adenylate cyclase (EC 4.6.1.1) in adipose tissue, and this enzyme in turn increases the concentration of adenosine 3' 5' monophosphate (cyclic 3' 5' AMP) which activates the lipase that splits triglyceride to produce fatty acid (Rizack, 1964, 1965; Butcher,

1966). The enzyme adenylate cyclase, which cyclizes ATP to cyclic 3' 5' AMP, is present in neural tissue, mainly in synaptic membranes (De Robertis *et al.*, 1967). Noradrenalin and 5-hydroxytryptamine were shown to increase the concentration of unesterified fatty acids in synaptosomes and synaptic membranes *in vitro* (Price and Rowe, 1972). Recently a phospholipase A2 system has been described in guinea-pig synaptic membranes, which is stimulated by neurotransmitters, putative neurotransmitters and by cyclic nucleotides (Gullis and Rowe, 1975 a, c). Electrical stimulation is known to cause an increase in 3' 5' cyclic AMP levels in brain (Kakiuchi *et al.*, 1969), and acetylcholine stimulates the formation of cyclic GMP in brain slices (Lee *et al.*, 1972; Kuo *et al.*, 1972). Much of the evidence to date suggests that under stimulated conditions, the increase in unesterified fatty acid levels is mainly due to the activation of a phospholipase A2 system, which is associated with synaptic membranes.

Unesterified fatty acids could conceivably arise as a result of phospholipase C activity combined with diacylglycerol and monoacylglycerol lipase activities. Phospholipase C (EC 3.1.4.3) is present in neural tissue, mainly associated with particulate fractions (Barnholz *et al.*, 1966; Thompson and Keough, 1972; Williams *et al.*, 1973). The presence of mono, di and triacylglycerol lipases in brain has already been mentioned. At the onset of ischaemia there is a rapid production of

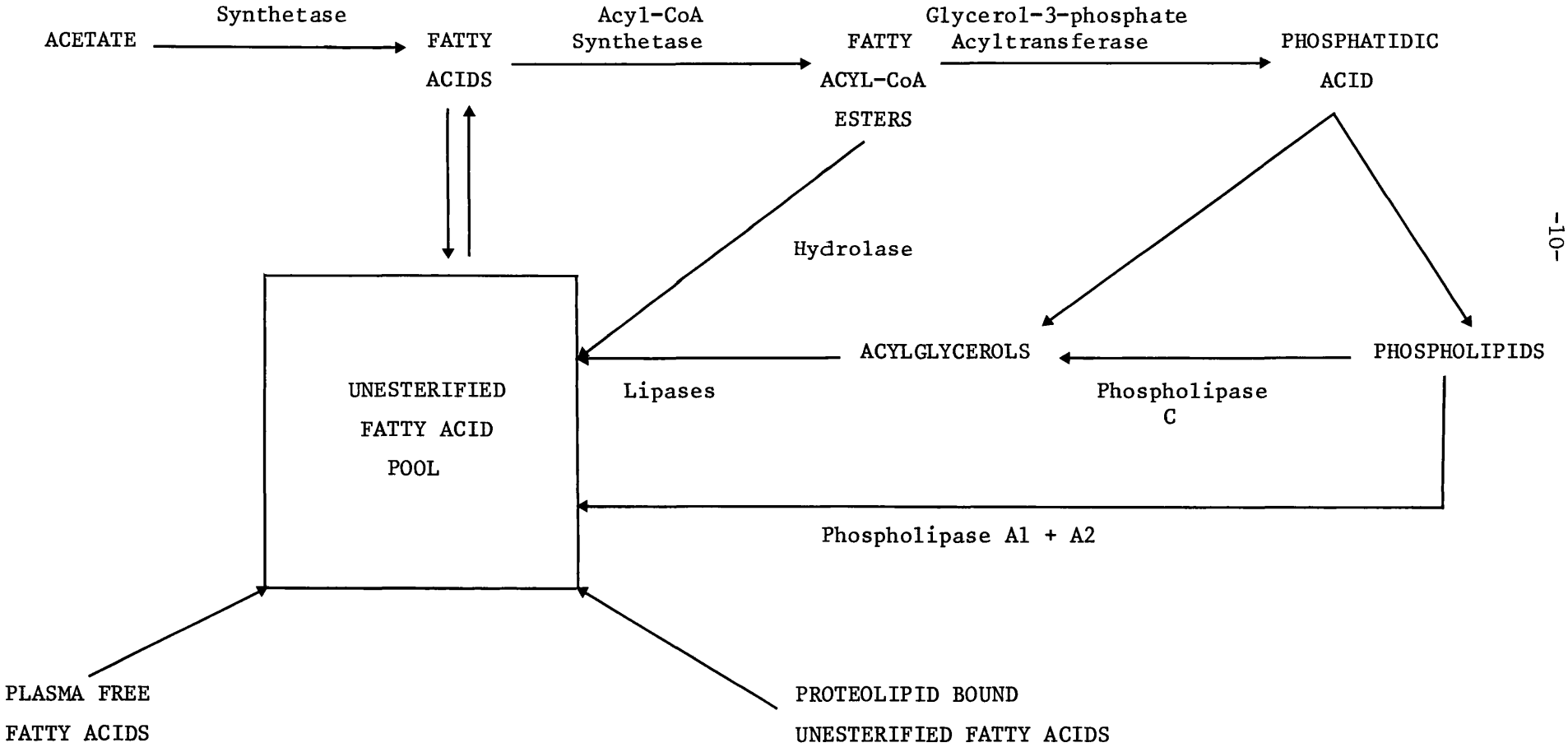
diacylglycerols and unesterified fatty acids rich in stearate, arachidonate and docosahexaenoate (Banschbach and Geison, 1974; Aveldano and Bazan, 1975). Hydrolysis of triacylglycerols cannot account for the appearance of arachidonate and the majority of the stearate and docosahexaenoate in the released diacylglycerols and unesterified fatty acids. Brain phospholipids are rich in stearate, arachidonate and docosahexaenoate (Ansell, Dawson and Hawthorne, 1973). The appearance of these fatty acids in the unesterified fatty acid and diacylglycerol pools, suggests that there may well be a phospholipase C activity coupled with a diacylglycerol lipase activity in brain, which could make a significant contribution to the increased levels of unesterified fatty acid that are found following electroshock and convulsant drug treatment. There is no evidence to suggest that phospholipase C is activated by cyclic nucleotides or neurotransmitters in a manner similar to phospholipase A2. However mono, di and triacylglycerol lipases from guinea-pig synaptic membranes are stimulated by neurotransmitters (Vyvoda and Rowe, 1973).

Unesterified fatty acids have been found associated with proteolipids in rat brain, and constitute a significant proportion of the total brain unesterified fatty acids (Lunt and James, 1976). The turnover of this pool of unesterified fatty acids is very slow, and it does not exchange readily with the main pool of fatty acids (James and Lunt, 1976).

Nothing is known about the release of proteolipid bound unesterified fatty acids under conditions of stimulation. The possible sources of unesterified fatty acids in the brain are summarized in Fig. 1.

The role of unesterified fatty acids in brain remains obscure. Recent investigations assign increasing importance to the non-polar acyl groups of brain membrane phospholipids, in relation to essential neuronal activities such as ion transport (Sun and Sun, 1974) and the synaptosomal uptake of noradrenalin (Sun, 1974). Thus the endogenous activity of enzymes promoting phospholipid deacylation may play an important role in the regulation of synaptic transmission-related events. Degradation of membrane phospholipids by phospholipase action would be expected to have gross effects on the integral structure of the membrane. A disturbance in the protein conformation of the plasma membrane after phospholipase A2 treatment has been related to changes in optical activity (Gordon *et al.*, 1969) and electron spin resonance (Simpkins *et al.*, 1971). The splitting of membrane phospholipids by phospholipase A2 is therefore associated with a disturbance in the normal relationship between the membrane proteins and lipids, which appears to be critical for the retention of membrane function.

FIG 1. Possible sources of unesterified fatty acids in brain



Guinea-pig synaptic membranes exhibit an acylase as well as a deacylase activity. The acylation of membrane phospholipids is essentially into position 2 of the glycerol backbone and is stimulated by neurotransmitters and cyclic nucleotides (Gullis and Rowe, 1975 b, c). Thus within synaptic membranes the stimulations of acylation and phospholipase A2 appear to be directly opposed. Both of these reactions must be controlled in some way. Calcium concentration may be one mechanism for control. In the presence of 3' 5' cyclic AMP high concentrations of Ca^{2+} stimulated acylation whilst inhibiting phospholipase A2. Low concentrations of Ca^{2+} in the presence of 3' 5' cyclic AMP stimulated phospholipase A2 (Gullis and Rowe, 1975c). Thus a high concentration of Ca^{2+} would control the direction of the stimulations by cyclic AMP by selectively switching off phospholipase A2.

Weller and Rodnight (1971) have shown that incubation of synaptic membranes in the presence of Mg^{2+}, with and without ATP, leads to a net phosphorylation and dephosphorylation respectively of membrane protein. Gullis and Rowe(1975c) have shown that preincubation of synaptic membranes with a mixture of ATP and $MgCl_2$ for short periods before the addition of substrate eliminated detectable hydrolysis by phospholipase A2 and stimulated subsequent acylation. Preincubation of membranes in the absence of ATP or $MgCl_2$ considerably enhanced the hydrolysis of phospholipids by phospholipase A2. These

observations suggest that a kinase-catalysed phosphorylation activates acylation or deactivates phospholipase A2, and that a phosphatase activates phospholipase A2 or deactivates acylation. Cyclic AMP activates phospholipase A2 in synaptic membranes (Gullis and Rowe, 1975c), and stimulation may be achieved through a cyclic AMP stimulated protein phosphatase, similar in nature to the phosphatase isolated from toad bladder (De Lorenzo and Greengard, 1973). Gullis and Rowe (1975c) have shown that inhibitors of protein phosphatase predisposed synaptic membranes to a stimulation of acylation. Furthermore the acylation of lysophosphatidylcholine had the characteristics of a kinase-mediated system insofar that ATP was required for the maximum rate of reaction, and that acylation was inhibited by adenosine, ADP and 5' AMP.

Synaptic membranes contain all the enzymes and substrates necessary for a cyclic AMP stimulated protein kinase and protein phosphatase system - adenylate cyclase and phosphodiesterase (De Robertis *et al.*, 1967), cyclic AMP activated protein kinase (Maeno *et al.*, 1971); Gaballah and Popoff, 1971), substrate for protein kinase (Johnson *et al.*, 1971; Ueda *et al.*, 1973) and protein phosphatase (Maeno and Greengard, 1972). Moreover phosphodiesterase is localized in post-synaptic nerve endings, most of it immediately adjacent to the synaptic membrane (Florendo *et al.*, 1971). Most of the evidence suggests that control of the phospholipase A2-acylation system

by phosphorylation-dephosphorylation, and by Ca^{2+}, may be the mechanism whereby changes in membrane permeability to cations are brought about.

Acetylcholine, noradrenalin, dopamine and 5-hydroxy-tryptamine stimulate the turnover of phosphatidylinositol in brain (Hokin, 1969, 1970; Abdel-Latif *et al.*, 1974; Friedel *et al.*, 1974; Lapetina and Michell, 1974; Widlund and Heilbronn, 1974; Lunt and Pickard, 1975). The stimulated cycle of breakdown and resynthesis appears to be localized mainly in the presynaptic terminals of synapses, rather than in the post synaptic cells. Breakdown was most marked in fractions rich in synaptic vesicles, presynaptic plasma membranes and small intact nerve endings (Lunt and Pickard, 1975), and the newly-synthesised phosphatidylinositol was directly localized in intact synaptosomes (Abdel-Latif *et al.*, 1974). Durrell, Garland and Friedel (1969) suggested that the primary effect of acetylcholine is to increase the breakdown of phosphatidyl-inositol to diglyceride by the action of phospholipase C. The presence of a phospholipase C in brain that is active towards phosphatidylinositol has been reported by Thompson and Keough (1972). Phosphatidylinositol is rich in stearate and arachidonate (Ansell, Dawson and Hawthorne, 1973). The action of a phospholipase C on synaptic membrane phosphatidylinositol would account for the increased levels of diglyceride rich in stearate and arachidonate that are found following the onset of

ischaemia (Banschbach and Geison, 1974; Aveldano and Bazan, 1975). Stimulated phospholipase C activity would certainly alter the properties of the synaptic membrane and may affect its permeability characteristics. It is not unreasonable to suppose that the membrane is opened up at specific areas rich in phosphatidylinositol by a phospholipase C enzyme specific for inositol phospholipids. This would be a more economical process than the destruction of all of the membrane phospholipids.

Both of the mechanisms described, which are believed to modify membrane function, involve a phospholipid degradation step, followed by a resynthesis step. The role of unesterified fatty acids in this sequence of reactions would presumably be in the latter step, the resynthesis of phospholipids.

It has been shown that there is a rapid incorporation of radioactively labelled acetate into brain unesterified fatty acids *in vitro* (Rowe, 1964, 1969; Lunt and Rowe, 1968; Carey, 1975a) and *in vivo* (Dhopeshwarkar *et al.*, 1969a, 1971c). Of the principle fatty acids isolated palmitate had the highest specific radioactivity (Rowe, 1964; Dhopeshwarkar *et al.*, 1971c). Labelled palmitate was synthesised *de novo* from acetate and stearate was formed from acetate by chain elongation (Dhopeshwarkar *et al.*, 1969a, 1971c). Unesterified fatty acids turnover very rapidly in the brain (Sun and Horrocks, 1971; Yau and Sun, 1973, 1974) with a half- life of

5-6 minutes. Radioactively labelled acetate and fatty acid are rapidly incorporated into acylglycerols and phospholipids (Rowe, 1969; Sun and Horrocks, 1971; Dhopeshwarkar *et al.*, 1971c; Yau and Sun, 1973, 1974; Carey, 1975a), which suggests that unesterified fatty acids are precursors for acylglycerol and phospholipid synthesis in the brain.

The incorporation of unesterified fatty acids into acylglycerols and phospholipids requires the activation of the fatty acids to their acyl-CoA esters. The enzyme responsible for this activation, palmitoyl-CoA synthetase is present in brain (Cantrill and Carey, 1975). The next step in the incorporation involves specific acyltransferases, which directly transfer the fatty acid moiety of the acyl-CoA esters to either lysophospholipids, glycerol-3-phosphate or monoacylglycerol-3-phosphate. The products of these enzymatic reactions are phospholipids, monoacylglycerol-3-phosphate and phosphatidic acids, respectively. Phosphatidic acids are important intermediates common to the biosynthesis of phospholipids, triacylglycerols and diacylglycerols. The presence of a glycerol-3-phosphate acyltransferase (EC 2.3.1.15) in brain microsomes has been reported by Carey (1975b), and it is known that brain homogenates and subcellular fractions are capable of acylating lysophospholipids (Webster and Alpern, 1964; Gullis and Rowe, 1975b,c).

In the work presented here the incorporation of radioactively labelled acetate into the three major pools of fatty acids in brain (ie. unesterified fatty acids, fatty acids from acylglycerols and fatty acids from phospholipids) has been examined. The half-life of the unesterified fatty acid pool was determined and in addition the turnover of the long-chain fatty acyl-CoA ester pool in brain has been measured. The role of the enzyme palmitoyl-CoA hydrolase in brain was also investigated. The results confirm the view that unesterified fatty acids are precursors for phospholipid and acylglycerol biosynthesis in brain, under non-stimulated conditions. It is suggested that under non-stimulated conditions, hydrolysis of long-chain fatty acyl-CoA esters could contribute to the maintenance of the unesterified fatty acid pool.

MATERIALS AND METHODS

MATERIALS

General chemicals were purchased from British Drug Houses Ltd., Poole, Dorset and from Fisons Ltd., Loughborough, Leicestershire. Acetone, chloroform, diethyl ether, light petroleum (b.p. 40-60°C) and methanol were redistilled before use. Where possible, analytical grade reagents were used. Special materials were purchased from the following suppliers.

The Boehringer Corporation (London) Ltd., Lewes, Sussex

Acetyl phosphate

Citrate synthase

Malate dehydrogenase

Nicotinamide adenine dinucleotide (NAD^+)

Phosphotransacetylase

British Drug Houses Ltd., Poole, Dorset

Acetylcholine chloride

Adrenalin

Carbamylcholine chloride

2,6-dichlorophenolindophenol

5-hydroxytryptaminecreatine sulphate

N-methyl-N-nitrosotoluene-4-sulphonamide.

L-noradrenalin

Phenolphthalein

Jones Chromatography Ltd., Llanbradach, Wales

Apiezon L grease

Celite 100-120 mesh

Merck, Darmstadt, Germany

Kieselgel 60 HR reinst nach Stahl-Silica gel-binder-free (supplied by British Drug Houses Ltd., Poole, Dorset).

New England Nuclear, Boston, U.S.A.

Palmitoyl Coenzyme A (Palmitoyl-1-^{14}C)

Packard Instruments Ltd., Caversham, England

2,5-diphenyl oxazole

P-LBiochemicals, Milwaukee, U.S.A.

Oleoyl Coenzyme A

Palmitoyl Coenzyme A (supplied by International Enzymes Ltd., Windsor, England)

Radiochemical Centre, Amersham, England

[1-^{14}C] acetate

Glycerol tri-[1-^{14}C] palmitate

[1-^{14}C] palmitic acid

Ralph N. Emanuel Ltd., Wembley, England

5,5'-dithiobis-(2-nitrobenzoic acid)

Sigma Chemical Company, London, England

Bovine serum albumin (fraction V).

Coenzyme A

Dithiothreitol

L-malic acid

Palmitic acid

Standard mixture of methyl tridecanoate, methyl tetradecanoate, methyl pentadecanoate, methyl hexadecanoate and methyl heptadecanoate.

Standard mixture of methyl octadecanoate, methyl nonadecanoate, methyl heneicosanoate, methyl eicosanoate and methyl docosanoate.

METHODS

1) EXPERIMENTAL MATERIALS

(i) Experimental animals

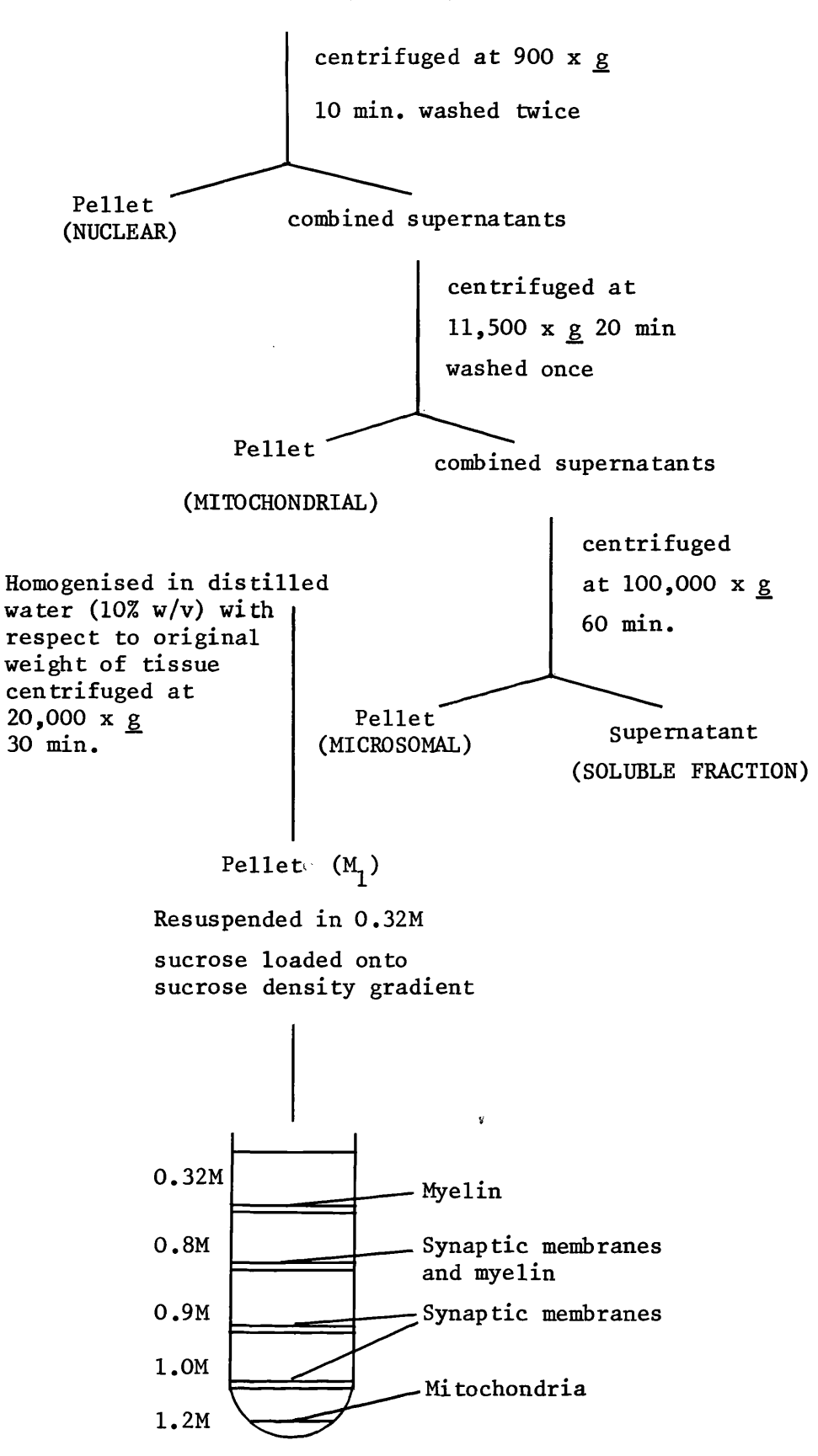
Adult male Wistar rats 150-200g, kept on a diet of Oxoid rat/mouse breeding pellets and water *ad libitum*, were used for all experiments on rat brain. Rats were killed by decapitation using a Luckham animal guillotine.

(ii) Tissue subcellular fractionation

Brain subcellular fractions were prepared using the method of Lapetina, Soto and De Robertis (1967).

Cerebral cortex substantially freed from white matter was homogenised in ice-cold 0.32M sucrose to give a tissue concentration of 10% (w/v). The homogenisation was carried out in a Potter-Elvehjem homogeniser, the teflon pestle having a radial clearance of 0.0075 in and rotating at 1,200 rev./min. A total of twenty up and down movements of the pestle were made in a total time of 2 min. The initial homogenate was then fractionated according to the scheme outlined in Fig. 2. Subcellular fractions separated on sucrose density gradients were removed by aspiration, diluted with distilled water and the tissue pelleted by centrifugation at 100,000 x g for 1 h.

Fig. 2. TISSUE FRACTIONATION SCHEME



2) EXTRACTION OF LIPIDS

Lipids were extracted by a modification of the method of Folch, Lees and Sloane-Stanley (1957), as described below.

Rat cerebral cortices and subcellular fractions were homogenised in 20 vol. of chloroform-methanol (2:1, v/v), (assuming 1 g of tissue has a volume of 1 ml). The homogenates were left to stand at room temperature for 20 min. The insoluble material was removed by filtration and washed twice with a further 10 vol. of chloroform-methanol (2:1, v/v). The extracts were combined and the insoluble material retained for protein analysis.

3) THE SEPARATION OF THE TWO MAIN LIPID CLASSES

Non-phosphorus containing lipids were separated from phosphorus-containing lipids by a solvent extraction procedure.

The combined chloroform-methanol extracts were evaporated to dryness under reduced pressure using a rotary evaporator. Water was removed by the repeated addition of methanol, and concentration to dryness. The dry lipid film was then extracted three times with 10 ml of acetone. The solution contained all the neutral lipids with a trace of some phosphorus-containing lipids. The material which was not extracted by acetone was composed essentially of phosphorus-containing lipids (Lovelock, James and Rowe, 1960; Lunt, 1967).

4) THE SEPARATION OF UNESTERIFIED FATTY ACIDS AND ACYLGLYCEROLS

Two procedures were employed to separate unesterified fatty acids from acylglycerols.

(i) Separation by thin-layer chromatography

Thin-layer plates were prepared from silica gel G, with an adsorbent layer thickness of 0.5 mm. The activation and development of the plates was carried out according to the method described by Skipski, Smolowe, Sullivan and Barclay (1965). The procedure involved activating the plates for 30 min at 110°C and washing them by ascending chromatography in chloroform-methanol (4:1, v/v) overnight. After washing the plates were dried and activated at 110°C for 30 min prior to use.

Loading of the plates: The neutral lipid extract was concentrated to dryness and redissolved in 5 ml of acetone. The solution was then evaporated down to a small volume (0.5 ml) under a stream of nitrogen. This solution was then applied to the plate in a band 3 cm wide, using a microlitre syringe. Marker spots of a solution of oleic acid in acetone were applied to the edges of the plates. The plates were chromatographed in the solvent system di-isopropyl ether-acetic acid (96:4, v/v). The solvent front was allowed to migrate a distance of 13 cm, the plates were dried at room temperature in an atmosphere of nitrogen for 1 h and then chromatographed in

the second solvent system, light petroleum (b.p. 40-60°C)-diethyl ether-acetic acid (90:10:1, by vol.), until the solvent front had migrated a distance of 19 cm. The plates were dried as before and the bands visualised using iodine vapour. The band corresponding to the unesterified fatty acid band was removed and extracted three times with 10 ml of diethyl ether. The bands which were identified as mono, di and triacylglycerols were also removed and extracted as above.

(ii) Separation by solvent extraction

The procedure used was a modification of the method described by Rowe (1964). The neutral lipid extract was concentrated to dryness under a stream of nitrogen and redissolved in 10 ml of water-ethanol (1:1, v/v). The solution was cooled to 0°C and 0.2 ml of glacial acetic acid added. The aqueous-ethanol extract was made just alkaline to phenolphthalein by the addition of 0.4 ml of 7.1N KOH and a few drops of 0.07N KOH. The mixture was then extracted three times with 5 ml of light petroleum (b.p. 40-60°C) to remove lipid esters. Where necessary emulsions promoted by soaps were separated by centrifugation at 0°C. After acidification by the addition of 0.4 ml of 10N sulphuric acid, the mixture was extracted again three times with 5 ml of light petroleum to collect the unesterified fatty acids. The lipid ester extract was composed essentially

of cholesterol, acylglycerols and cholesterol esters.

5) THE EXTRACTION OF FATTY ACIDS FROM PHOSPHOLIPIDS AND ACYLGLYCEROLS

Phospholipids and acylglycerols were hydrolysed by the procedure of Lovelock, James and Rowe (1960).

Hydrolysis of phospholipids: The residue from acetone extraction was dissolved in a mixture of aqueous 50% (v/v) ethanol (5 ml) and aqueous 40% (w/v) KOH (1 ml) and the mixture heated at 60°C for 4 h. After the mixture had cooled to room temperature, 3 ml of water, 4 drops of glacial acetic acid and 2 ml of 10N sulphuric acid were added and the solution thoroughly mixed. The mixture was then extracted three times with 5 ml of light petroleum (b.p. 40-60°) to collect the fatty acids.

Hydrolysis of acylglycerols: The acylglycerol extract was concentrated to dryness under a stream of nitrogen and redissolved in a mixture of ethanol (5 ml) and aqueous 40% (w/v) KOH (1 ml). The mixture was then heated at 60°C for 4 h. When the mixture had cooled, water (3 ml) and glacial acetic acid (4 drops) were added and the hydrolysate extracted three times with light petroleum (5 ml) to remove unsaponified lipid. Sulphuric acid (10N; 2 ml) was added and the mixture again extracted with light petroleum as before, to obtain the fatty acids.

6) THE ESTIMATION OF FATTY ACIDS BY GAS-LIQUID CHROMATOGRAPHY

Fatty acids have been estimated as their methyl esters by gas-liquid chromatography in all of the experiments presented in this work. The estimation of fatty acids by this procedure has many advantages over other methods of estimation, such as direct weighing, titration against alkali, colourimetric determinations and photodensitometry measurements of charred material following separation by thin-layer chromatography. Gas-liquid chromatography has a very high sensitivity enabling 1 μg of an individual methyl ester to be easily detected. In addition to a quantitative estimation of fatty acids the method also separates a mixture of components, allowing a determination of the distribution of the fatty acids to be made.

(i) Apparatus

All samples of methyl esters of fatty acids were analysed using a Pye series 104 gas chromatograph fitted with a flame ionization detector. The samples were chromatographed at 200°C on a glass column (1.5 m x 3 mm) packed with 100-120 mesh Celite coated with 5% (w/w) Apiezon L grease. The column packings were prepared as described by James (1960). The flow rate of the carrier gas nitrogen was maintained at 60 ml per min for all determinations.

(ii) The preparation and loading of samples

Prior to estimation all samples of fatty acid methyl

esters were made up to a volume of 5 ml in light petroleum (b.p. 40-60^{o}C), and known volumes of this solution were used for each determination. To each sample was added a known amount of methyl heptadecanoate. This was kept as a solution in light petroleum of known concentration. The combined sample and standard were evaporated to a small volume (25 µl) in a 10 ml pointed glass centrifuge tube under a stream of nitrogen. A small amount of the concentrated sample (2-3 µl) was then loaded onto the column using a microlitre syringe.

Incorporation of the standard solution of methyl heptadecanoate enabled the quantity of each methyl ester in the sample to be determined by comparison of their respective peak areas with that of methyl heptadecanoate (Rowe, 1964). The areas of the peaks obtained on the recorder chart were determined by triangulation. The quantity of methyl heptadecanoate added to each sample was such that the peak area was of the same order of magnitude as the peaks of the methyl esters in the sample.

(iii) The identification of the methyl esters

Methyl esters of fatty acids were identified by comparison of their retention times with those of authentic standards. This enabled methyl esters of fatty acids with chain lengths ranging from C13 to C22 to be readily identified.

The Apiezon L column separates methyl esters primarily on the basis of chain length and also separates a monounsaturated methyl ester from its corresponding saturated ester (James, 1960).

7). THE PREPARATION OF FATTY ACID METHYL ESTERS

Fatty acids were converted to their corresponding methyl esters by treatment with diazomethane, prepared by the method of Vogel (1957). In all cases diazomethane was freshly prepared and triply distilled immediately before use. The fatty acids extracted in light petroleum (b.p. 40-60^{o}C) were concentrated to dryness under a stream of nitrogen and redissolved in 5 ml of diethyl ether. A solution of diazomethane in diethyl ether was then added dropwise until the yellow colour of the diazomethane persisted. The solution was left to stand at room temperature for 20 min. The excess diazomethane was then removed by distillation. The resulting solution of methyl esters in diethyl ether was evaporated to dryness under a stream of nitrogen and redissolved in light petroleum (b.p. 40-60^{o}C).

8). THE REMOVAL OF UNMETABOLIZED RADIOACTIVELY LABELLED ACETATE FROM FATTY ACID EXTRACTS

Several methods were tried in an attempt to remove unmetabolized [1-^{14}C] acetate from the fatty acid extracts.

(i) Washing of the tissue homogenate

Rat cerebral cortices were homogenised for 5 min in 5 ml of ice-cold 0.14M barbital-acetate buffer, pH 7.4 (Michaelis, 1931). A further 20 ml of ice-cold buffer were added to the homogenate and the tissue precipitated by centrifugation at 60,000 x g for 10 min. The precipitate was washed twice by resuspending in a further 20 ml of ice-cold buffer and sedimenting the tissue as before. The temperature was maintained at 0-4°C throughout the washing procedure. The barbital-acetate buffer was prepared by mixing 50 ml of solution A (see below) with 50.5 ml of solution B and 20 ml of solution C and making the final volume of the mixture up to 250 ml with distilled water.

Solution A: 19.43 g of sodium acetate and 29.45 g of sodium barbital dissolved in 1 *l* of water.

Solution B: 0.1 N hydrochloric acid.

Solution C: 8.5% (w/v) sodium chloride solution.

The washed tissue pellet was extracted with 20 volumes of chloroform-methanol (2:1, v/v) using the method described earlier.

(ii) Washing of the total lipid extract

The method used was based on the procedure described by Clayton and Rowe (1966).

The chloroform-methanol extract was washed once with 0.2 vol. of 0.1M sodium acetate solution and the upper layer removed by aspiration. The lower layer was washed twice with 'theoretical upper phase' (Folch *et al.*, 1957) containing chloroform-methanol-0.5M sodium acetate solution (3:48:47, by vol.). The volume of upper phase was equal to that of the aqueous phase removed in the first washing. Any emulsions which formed during the washing procedure were separated by centrifugation at 0°C. The washed total lipid extract was then separated into its two main classes by the procedure described previously.

(iii) Silicic acid chromatography

The light petroleum fatty acid extracts were dried, by filtering through anhydrous magnesium sulphate, concentrated to dryness under a stream of nitrogen and redisssolved in 2 ml of diethyl ether. Silicic acid was activated by heating at 110°C for 3 h, and a silicic acid column prepared by slurrying 2 g of the silicic acid with diethyl ether and pouring the slurry into a 1 cm diameter glass column, the bottom end of which was plugged with glass wool. The solution of fatty acids, in 2 ml of diethyl ether, was applied to the column as the last of diethyl ether used in packing the column passed into the silicic acid. The fatty acids were eluted by the application of a further 30 ml of diethyl ether to the column. The eluted fatty acids were evaporated to dryness under a stream of nitrogen, redissolved in 5 ml of diethyl ether and

treated with diazomethane as previously described.

(iv) Treatment with methyl acetate

Treatment of fatty acid extracts, which contain unmetabolized [1 - ^{14}C] acetate, with diazomethane produces fatty acid methyl esters and methyl [1 - ^{14}C] acetate. In an attempt to remove the contaminating methyl [1 - ^{14}C] acetate 5 ml of methyl acetate was added to the methyl ester fractions and then removed by rotary evaporation. The residue was distilled to dryness a further four times with 5 ml of methyl acetate. The fatty acid methyl esters were then dissolved in 5 ml of light petroleum (b.p. 40-60^{o}C) prior to estimation by gas-liquid chromatography.

The most efficient method for removing unmetabolized radioactively labelled acetate proved to be a combination of methods (i) and (ii), i.e. washing of the tissue homogenate followed by a washing of the total lipid extract (see results section).

9). ESTIMATION OF LONG-CHAIN FATTY ACYL-CoA ESTERS IN BRAIN

The procedure used was a modification of the method described by Lee and Fritz (1972). Long-chain acyl-CoA esters were measured by determining the CoA-SH liberated following alkaline hydrolysis of the long-chain acyl-CoA esters precipitated by perchloric acid.

(i) Precipitation of long-chain acyl-CoA esters.

Rat brains were rapidly removed and the cerebral hemispheres frozen in powdered solid carbon-dioxide. The frozen cerebral hemispheres were homogenised for 5 min in 15 ml of ice-cold 30% (w/v) perchloric acid. Homogenisation was carried out in a Potter-Elvehjem homogeniser using the conditions described previously. The homogenate was centrifuged at 100,000 x g for 30 min and the tissue pellet washed once by resuspending in a further 15 ml of ice-cold 30% (w/v) perchloric acid and centrifuging as before. The tissue pellet contained the perchloric acid insoluble long-chain acyl-CoA esters.

(ii) Hydrolysis of long-chain acyl-CoA esters.

The tissue pellet was resuspended in 5 ml of ice-cold 15mM dithiothreitol solution and the suspension adjusted to pH 11-12 with a few drops of 6M KOH. The mixture was then heated at 55-60^{o}C for 15 min after which 0.5 ml of perchloric acid (70%, w/v) was added. The hydrolysate was centrifuged at 100,000 x g for 15 min and the supernatant fraction neutralized with KOH. The CoA-SH content of the supernatant was then determined using the method described below.

(iii) Estimation of CoA-SH.

CoA-SH was assayed by a modification of the Allred and Guy procedure (1969), described by Lee and Fritz (1972). The following solutions were prepared:-

0.3M potassium chloride solution dissolved in 1.5 M Tris-HCl buffer, pH 7.2

0.1M L-malate

0.1 M acetyl phosphate

6 mM nicotinamide adenine dinucleotide (NAD)

20 mM dithiothreitol.

Equal volumes (2 ml) of the reagents, which were stable for weeks at refrigerator temperatures, were combined in a premix just prior to use. The CoA-SH sample (0.5 ml) was added to a cuvette of 1 cm light path which contained 0.5 ml of the premix. Malate dehydrogenase (EC 1.1.1.37), (10 units) and citrate synthase (EC 4.1.3.7), (2.8 units) were added and the mixture incubated at 30°C for 2 min. After incubation phosphotransacetylase (EC 2.3.1.8), (20 units) was added to start the reaction, and the formation of NADH was followed at 340 nm using a Pye Unicam model 1800 spectrophotometer fitted with a Unicam AR25 linear chart recorder. A standard curve in the concentration range 0 - 1.5 nmoles was prepared using a solution of CoA-SH. The standard CoA-SH solution was prepared by dissolving 9 mg of CoA-SH in 10 ml of water to give a concentration of 1 mM, and diluting this with water to give two solutions, one with a concentration of 5 nmoles of CoA-SH per ml and the other a concentration of 1 nmole of CoA-SH per ml.

10). EXTRACTION OF RADIOACTIVELY LABELLED LONG-CHAIN FATTY ACYL-CoA ESTERS

Several methods were used in an attempt to isolate long-chain

fatty acyl-CoA esters free from contaminating [1 - ^{14}C] acetate and radioactively labelled lipids.

Method (a)

This procedure was based on the method of Denton and Halperin (1968).

Rat cerebral hemispheres were removed and homogenised in 10 ml of ice-cold 5% (w/v) perchloric acid in the manner described earlier. The homogenate was centrifuged at 100,000 x g for 30 min to precipitate the tissue and insoluble long-chain acyl-CoA esters. The pellet was washed twice, to remove contaminating [1 - ^{14}C] acetate, by resuspending in a further 10 ml of ice-cold perchloric acid and precipitating the insoluble material by centrifugation as before. The pellet was resuspended in 5 ml of 5% (w/v) perchloric acid and extracted three times with 10 ml of peroxide-free diethyl ether to remove any lipids. Excess diethyl ether was removed by bubbling the solution with nitrogen. The insoluble material was precipitated by centrifugation at 100,000 x g for 30 min and resuspended in 5 ml of 15 mM dithiothreitol. The insoluble long-chain acyl-CoA esters were hydrolysed and the liberated CoA-SH determined according to the methods described previously. The recovery of long-chain fatty acyl-CoA esters was poor and the radioactive labelling pattern inconsistent. This suggested that the diethyl ether wash destroyed much of the acyl-CoA esters and that radioactively labelled material was contaminating the acyl-CoA ester fraction.

Method (b)

This method was based on the washing procedure described by Ullman and Radin (1972).

Rat cerebral hemispheres were homogenised in 10 ml of ice-cold 30% (w/v) perchloric acid, centrifuged and the tissue pellet washed twice with a further 10 ml of perchloric acid, according to the methods described earlier. The tissue pellet was resuspended in 3 ml of 0.58% NaCl solution, containing 15 mM dithiothreitol, and the suspension adjusted to pH 5 with 6M KOH. Chloroform-methanol (2:1, v/v), (15 ml) was added and the solution thoroughly mixed. The mixture was centrifuged to separate the emulsion and the upper layer removed. Theoretical upper phase (Folch *et al.*, 1957), equal in volume to the upper phase removed in the first wash, was added and the solution mixed and centrifuged as before. The upper layer was removed and the combined upper layers acidified with 0.5 ml perchloric acid (70%, w/v). The acid insoluble material, free from contaminating lipids, was pelleted by centrifuging at 100,000 x $\underline{g}$ for 1 h and resuspended in 5 ml of 15 mM dithiothreitol. The resuspended material was then hydrolysed and any CoA-SH liberated determined in the manner described earlier. No CoA-SH could be detected in the supernatant fraction following hydrolysis, suggesting that the acyl-CoA esters were either destroyed during the isolation procedure, or that they were not extracted into the theoretical upper phase.

Method (c)

The technique used was based on the thin-layer chromatographic procedure described by Ullman and Radin (1972).

Rat cerebral hemispheres were homogenised in 10ml of ice-cold 30% (w/v) perchloric acid, centrifuged and the resulting pellet washed twice with a further 10 ml of perchloric acid, to remove any contaminating [1 - ^{14}C] acetate, as described previously. The washed tissue pellet was resuspended in 5ml of 15 mM dithiothreitol and the mixture adjusted to pH 6 with 6M KOH to solubilize the long-chain acyl-CoA esters. The insoluble material was precipitated by centrifugation at 100,000 x g for 1 h and the resulting supernatant lyophilized overnight. Oxalate-silica gel thin-layer plates were prepared from binder-free silica gel, with an adsorbant layer thickness of 0.5mm. The silica gel was shaken vigorously with 1% (w/v) potassium oxalate solution in the proportions 1:2 (w/v) for 2 min. The resulting slurry was applied to the plates using a Quickfit thin-layer plate spreader. The plates were left to dry and activated by heating at 110^{o}C for 1 h prior to use. The lyophilized powder was redissolved in 1 ml of 40 mM phosphate buffer pH 6 and this solution was applied to the plate in a band 5 cm wide using a microlitre syringe. Marker spots of a solution of authentic oleoyl-CoA were applied to the edges of the plate and the chromatogram developed in the solvent system chloroform-methanol-

water (45:45:15, by vol.). The plate was air-dried and the bands developed using iodine vapour. The band corresponding to the long-chain acyl-CoA esters was removed and resuspended in 5 ml of 15 mM dithiothreitol. The acyl-CoA esters were hydrolysed and the released CoA-SH determined using the methods described earlier. The method was successful in separating long-chain fatty acyl-CoA esters from contaminating lipids, however all the techniques which were used to visualize the acyl-CoA ester band, either destroyed the thioesters, making CoA-SH determination impossible, or resulted in the disintegration of the silica gel coating.

Method (d)

The extraction procedure used was essentially the method described by Tubbs and Garland (1964).

Rat cerebral hemispheres were homogenised in 10 ml of ice-cold 5% (w/v) perchloric acid and the tissue pellet washed twice to remove contaminating [1 - ^{14}C] acetate, in the manner described earlier. The washed tissue pellet was homogenised for 2 min at 40 - 45^{o}C with 30 ml of 0.2M phosphate buffer pH 6, containing bovine serum albumin (10 mg/ml), and the residue was recovered by centrifuging at 13,000 x g for 10 min. This extraction procedure was repeated three times in all. The pooled supernatants were acidified with 3 ml of perchloric acid (60%, w/v) and the insoluble material pelleted by

centrifugation as before. The precipitate was washed with 20 ml of acetone and extracted three times by homogenisation for 2 min at 45°C with 20 ml of propan-2-ol-pyridine-water (1:1:1, by vol.). The combined extracts were rapidly (in less than 20 min) dried in a rotary evaporator under nitrogen at reduced pressure. The dry residue was suspended in 40 ml of 40 mM phosphate buffer pH 6, kept on ice for 5 min and then centrifuged as before. The resulting supernatant was acidified with 2 ml of 12N hydrochloric acid and centrifuged for 10 min at 13,000 x g. The small precipitate was washed with 20 ml of acetone, centrifuged and then washed with 20 ml of peroxide-free diethyl ether. The washed material was precipitated by centrifugation, and after drying in a stream of nitrogen the residue was resuspended in 5 ml of 15 mM dithiothreitol solution. The resuspended material was hydrolysed and any CoA-SH liberated determined as described previously. No CoA-SH could be detected using this procedure, which suggested the long-chain acyl-CoA esters had been destroyed during the isolation procedure.

McGee and Spector (1975) using radioactively labelled lipid standards, reported that any method which quantitatively separated all lipids from long-chain fatty acyl-CoA esters also removed or destroyed much of the acyl-CoA esters. These workers developed a technique for separating acyl-CoA esters from the majority of the other lipids. The method , described below, is based on a thin-layer chromatographic procedure.

Method (e)

The procedure used was a modification of the method described by McGee and Spector (1975).

Rat cerebral hemispheres were rapidly removed and homogenised in 10 ml of ice-cold chloroform-methanol (2:1, v/v). The insoluble tissue was removed by centrifugation and the chloroform-methanol extract decanted off. The tissue pellet was extracted further by homogenising in 5 ml of ice-cold chloroform-methanol-water (7:7:3, by vol.). The tissue extracts were combined and evaporated to dryness under nitrogen using a rotary evaporator. Water was removed by the procedure described previously, and the dry film extracted with 2 ml of chloroform-methanol-water (7:7:3, by vol.). Thin-layer plates were prepared from silica gel G with an adsorbent layer thickness of 0.75 mm, and were activated by heating at 110^{o}C for 1 h immediately before use. The acyl-CoA extract was applied to the plate in a band 15 cm wide using a microlitre syringe. The plate was then developed in the solvent system chloroform-methanol-acetic acid-water (50:25:7:3, by vol.). The plate was air-dried to remove residual solvents and the silica gel, in a band 0.5 cm either side of the origin, scraped off. The gel was washed three times, to remove contaminating [1 -^{14}C] acetate, by suspending in 8 ml of 5% (w/v) perchloric acid and precipitating the insoluble material by centrifugation. The precipitated material was resuspended in 5 ml of 15 mM dithiothreitol solution and the

long-chain acyl-CoA esters hydrolysed as described earlier. The hydrolysate was cooled to $0^{o}C$, acidified with 0.5 ml of 70% (w/v) perchloric acid and centrifuged to remove insoluble material. The resulting supernatant was neutralized with 6M KOH and the CoA-SH content determined as previously described.

Of the five procedures which were tried this method gave the most consistent results* and was used to examine the incorporation of [1 -^{14}C] acetate into long-chain acyl-CoA esters.

11). RADIOACTIVE LABELLING OF FATTY ACIDS AND LONG-CHAIN FATTY ACYL-CoA ESTERS *IN VIVO*

(i) Radioactive precursors

The sodium salt of [1 -^{14}C] acetic acid, specific radioactivity 59 mCi/mmole, was used as the isotopically labelled precursor in all experiments. The sodium [1 - ^{14}C] acetate was dissolved in water to give a solution having an activity of 0.1 mCi/ml. [1 -^{14}C] palmitic acid, specific radioactivity 59 mCi/mmole, was dissolved in diethyl ether to give a final activity of 0.5 mCi/ml. Glycerol tri-[1 - ^{14}C] palmitate, specific radioactivity 46 mCi/mmole, was dissolved in diethyl ether to give a final activity of 2.5 µCi/ml. Radioactively labelled palmitic acid and glycerol tripalmitate were used to determine the recovery of unesterified fatty acids and acylglycerols following the two washing procedures described previously.

* Consistent in terms of recovery of acyl-CoA esters.

(ii) Labelling *in vivo*

Rats received 20 µl (2µCi) of the aqueous solution of [1 - ^{14}C] acetate by injection into the right lateral ventricle under light diethyl ether anaesthesia, using the procedure described by Noble *et al.* (1967). The rats were decapitated at varying time intervals after injection and the brains rapidly removed. In all experiments where the labelling of fatty acids was examined, the cerebral cortex was substantially freed from white matter before lipid extraction. Long-chain fatty acyl-CoA esters were extracted from whole cerebral hemispheres.

(iii)Counting of samples

Unesterified fatty acids were counted as their methyl esters. An aliquot (1 ml) of the methyl ester solution, dissolved in 5 ml of light petroleum (b.p. 40 - 60°C) was placed in a cylindrical screw-cap scintillation vial and 10 ml of scintillation phosphor added. The phosphor contained 2,5-diphenyl oxazole (5 g/l) as the scintillator and Triton X 100 (30%, v/v), dissolved in scintillation grade toluene.

Long-chain fatty acyl-CoA esters were estimated by counting the fatty acids released after alkaline hydrolysis. An aliquot (0.5 ml) of the hydrolysate was placed in a cylindrical screw-cap scintillation vial and 10 ml of scintillant added, as described above. All the samples were counted for 10 min or 10,000 counts in a Packard liquid scintillation spectrometer

model 3385. A background count was made for each experiment. This consisted of 10 ml of scintillation phosphor in a scintillation vial. The count obtained for the background sample was subtracted from that of each of the experimental samples. All counts were corrected to 100% efficiency.

12). PALMITOYL-CoA HYDROLASE ACTIVITY

Long-chain fatty acyl-CoA hydrolase activity was measured in a high-speed supernatant fraction of rat cerebral cortex, prepared as described by Kurooka *et al.* (1972).

Rat cerebral cortices were homogenised in 5 vol. of ice-cold 50 mM phosphate buffer pH 7 (assuming 1 g of tissue has a volume of 1 ml). The homogenate was centrifuged at 17,500 x g and the supernatant assayed for activity as described below.

Enzyme assays

Two assay procedures were used to determine the long-chain fatty acyl-CoA hydrolase activity in the high-speed supernatant.

(i) DTNB assay

The assay procedure used was a modification of the technique described by Kurooka *et al.* (1972). The following solutions were prepared :-

1mM 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB)
solution dissolved in 0.25 M tris-HCl buffer pH 8.

1mM palmitoyl-CoA solution.

Both solutions were stored frozen, in darkened bottles. 0.5 ml of the DTNB reagent was added to a cuvette of 1 cm light path which contained 0.5 ml of distilled water and 2 µl (approximately 5 milliunits) of supernatant. After incubation for 2 min at 30°C the reaction was started by adding 20 µl (20 nmoles) of palmitoyl-CoA and the formation of the yellow coloured product 5-thio-2-nitrobenzoic acid (E_{412}, 13,400 at pH 8) was recorded at 412 nm using a Pye Unicam model 1800 spectrophotometer. One unit of palmitoyl-CoA hydrolase is defined as the activity required to hydrolyse 1 µmole of palmitoyl-CoA per min under the conditions described.

(ii) DCPIP assay

This was essentially the assay procedure described by Anderson and Erwin (1971). The determination of enzyme activity was performed at 30°C in a 1.0 ml reaction mixture containing 5 µM palmitoyl-CoA, 0.001% (w/v) 2, 6-dichlorophenolindophenol and 0.05 M phosphate buffer. The reaction mixture was incubated for 2 min and the reaction started by the addition of 2 µl (approximately 0.5 milliunit) of the supernatant, which had been diluted ten times with 50 mM phosphate buffer pH 7.0. The change in optical density with respect to time was recorded using a Pye Unicam model 1800 spectrophotometer. A molar extinction coefficient of 19,000 at 600 nm was assumed for 2,6-dichlorophenolindophenol.

13) PROTEIN DETERMINATION

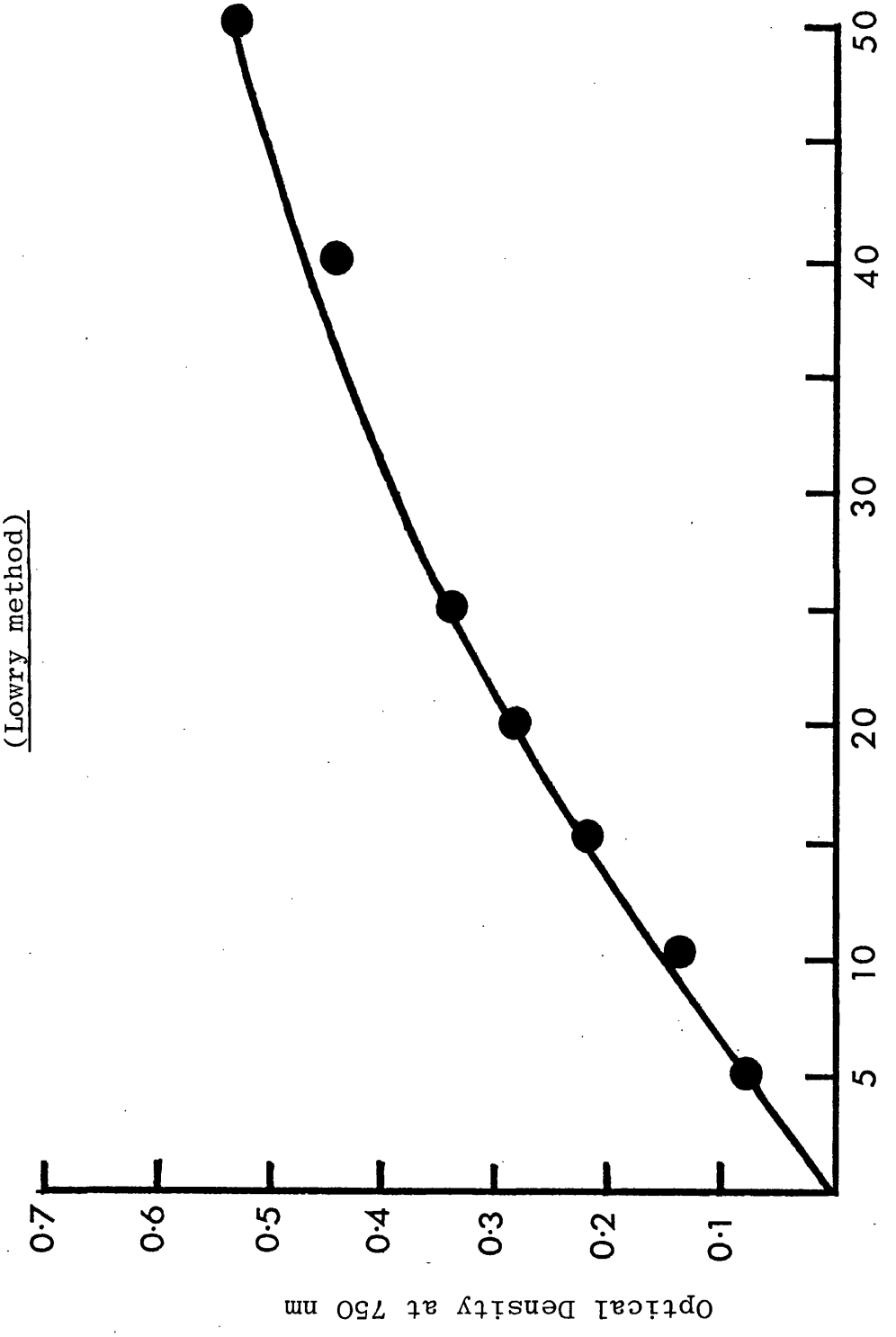
The method of Lowry *et al.* (1951) was used to determine the protein content of the tissue residue remaining after lipid extraction. The residue was dissolved in 50 ml of 2N NaOH by warming at 30°C for several days. The resulting solution was diluted with water to give a final normality of 0.5N, and this solution was subjected to the colourimetric procedure described by Lowry *et al.* A standard curve was prepared using a solution of bovine serum albumin (fraction V) in 0.5N NaOH solution, containing 1 mg protein per ml. The standard curve is shown in Fig. 3.

The tissue precipitated by perchloric acid during the estimation of long-chain acyl-CoA levels, was not readily soluble in 2N NaOH. Long-chain acyl-CoA levels were therefore expressed in nmoles per g wet weight. The rapid removal of the brains followed immediately by freezing in solid carbon dioxide meant that the tissue could not be weighed directly. Tissue wet weights were therefore calculated from the weight of the dry material which remained after the estimation of acyl-CoA ester levels, as described below.

The cerebral hemispheres from 4 rats were removed and their wet weights measured. The tissue was then treated in the manner described in section 9(i+ii). The insoluble material which remained was dried to a constant weight in an oven at 100°C.

The weight of the dry material was then expressed as a percentage of the original fresh tissue. Using this figure the dry weight of the experimental material which remained could be converted into grams of fresh tissue.

Fig. 3 Protein estimation standard curve (Lowry method)



RESULTS

SECTION 1

THE REMOVAL OF UNMETABOLIZED RADIOACTIVELY LABELLED ACETATE FROM FATTY ACID EXTRACTS

Preliminary experiments showed that at short incorporation times a considerable amount of unmetabolized [1 - ^{14}C] acetate is present in the excised cerebral cortex and this leads to contamination of the isolated fatty acid fractions. Consequently a number of procedures were tried in an attempt to remove the unmetabolized acetate, as described in Methods 8. A series of control experiments were carried out to establish the degree of contamination of the lipid fractions with [1 - ^{14}C] acetate.

(i) Treatment with methyl acetate.

The cerebral cortices from four non-injected rats were removed and homogenised in 20 vol of chloroform-methanol. Sodium [1 -^{14}C] acetate, (20 µl; 2 µCi) was then added to the homogenate and the unesterified fatty acids isolated and converted to their methyl esters by treatment with diazomethane. The fatty acid methyl esters were then treated with methyl acetate as described in Methods 8 (iv). The amount of radioactivity recovered in the unesterified fatty acid methyl ester fraction before and after treatment with methyl acetate is shown in Table 1.

TABLE 1. RECOVERY OF [1 - ^{14}C] ACETATE IN THE UNESTERIFIED FATTY ACID METHYL ESTER FRACTION

	TOTAL RADIOACTIVITY (d.p.m.)	
Rat	Before Treatment	After Treatment
1	6719	6541
2	7316	7218
3	7216	7015
4	7104	6919

This represents a net reduction in radioactivity of 2.34 ± 0.33% (mean ± S.E. 4 determinations).

(ii) Silicic acid chromatography

Sodium [1 - ^{14}C] acetate was added to the chloroform-methanol homogenates as described in (1) and the unesterified fatty acids isolated as described in Methods 4(i). The unesterified fatty acid extracts were subjected to silicic acid column chromatography in an attempt to remove contaminating [1 - ^{14}C] acetate as described in Methods 8(iii). The amount of radioactivity recovered in the unesterified fatty acid fraction, before and after silicic acid chromatography, is shown in Table 2.

TABLE 2. RECOVERY OF [1 - ^{14}C] ACETATE IN THE UNESTERIFIED FATTY ACID FRACTION.

	TOTAL RADIOACTIVITY (d.p.m.)	
Rat	Before Column Chromatography	After Column Chromatography
1	6954	5106
2	7218	5140
3	7004	5217
4	7103	5102

This represents a net reduction in radioactivity of 27.26 ± 0.74% (mean ± S.E. 4 determinations).

(iii)Washing procedures.

Cerebral cortices were removed from non-injected rats and homogenised in ice-cold 0.14M-acetate-barbital buffer. Sodium [1 - ^{14}C] acetate (20 µl; 2 µCi) was then added to the homogenate which was subjected to the washing procedures described in Methods 8 (i) and (ii).
The amount of radioactivity recovered in the fatty acid fractions is shown in Table 3.

TABLE 3. RECOVERY OF [1 - ^{14}C] ACETATE IN FATTY ACID FRACTIONS FROM RAT CEREBRAL CORTEX LIPIDS.

FRACTION	TOTAL RADIOACTIVITY (d.p.m.)
Initial homogenate	4.4×10^6
Unesterified fatty acids	254 ± 19 (5)
Neutral glyceride fatty acids	155 ± 5 (3)
Phospholipid fatty acids	110 ± 14 (3)

All values are given as mean ± S.E. (no. of experiments)

It was found that no further decreases in the levels of radioactivity of the fatty acid fractions were obtained by increasing the number of washes of either the tissue homogenate or of the lipid extracts.

(iv) Recovery of lipids

Experiments were done to establish the losses of unesterified fatty acids and of neutral glycerides incurred in the washing procedures. [1 - ^{14}C] palmitic acid (100 µl; 0.25 µCi) and glycerol tri- [1 -^{14}C] palmitate (100 µl; 0.25 µCi) were added to homogenates of cerebral cortex from individual rats. The homogenates were then subjected to the washing procedures described in Methods 8 (i) and (ii). The recovery of unesterified fatty acids and neutral glycerides is shown in Table 4.

TABLE 4. RECOVERY OF UNESTERIFIED FATTY ACIDS AND NEUTRAL LIPIDS

WASHING PROCEDURE	% RECOVERY UNESTERIFIED FATTY ACID	NEUTRAL GLYCERIDE
3 x washes with Barbital-acetate buffer	81 ± 0.9 (3)	85 ± 1.3(3)
3 x washes with Theoretical upper phase	77 ± 3.5 (3)	80 ± 0.9(3)

Results are expressed as mean ± S.E. (no. of experiments)

Experiments were carried out to determine the efficiency of the solvent extraction method used in separating unesterified fatty acids and neutral glycerides. Two groups of three rats were killed and the cerebral cortices removed and homogenised in barbital-acetate buffer. Radioactively labelled palmitic acid was added to one batch of homogenates and labelled glycerol tripalmitate was added to the other batch. The homogenates were washed (see Methods 8(i) and (ii)) and unesterified fatty acids and neutral glycerides isolated according to the solvent extraction procedure described in Methods 4 (ii). The overall recovery of unesterified fatty acids and neutral glycerides was 61%[1.] and 79%[2.] respectively. Less than 0.1% of the total neutral glyceride was recovered in the unesterified

1. 61·33 ± 1·45 %(3) 2. 79·0 ± 2·31 % (3).
Results are expressed as mean ± S.E. (no. of expts).

fatty acid fraction and only 1.3% of the total unesterified fatty acid was found in the neutral glyceride fraction.

SECTION 2

UNESTERIFIED FATTY ACID METABOLISM IN RAT CEREBRAL CORTEX *IN VIVO*

(i) Yield and composition of unesterified fatty acids.

Experiments were carried out in order to establish the yield and composition of the unesterified fatty acids from rat cerebral cortex. Unesterified fatty acids were isolated by the solvent extraction procedure described in Methods 4 (ii), and were estimated as their methyl esters by gas-liquid chromatography.

(a) Yield of unesterified fatty acid

The yield of unesterified fatty acid was 9.6 ± 0.35 μmoles/g protein (mean ± S.E. 64 determinations).

(b) Composition of unesterified fatty acids

The composition of the isolated unesterified fatty acids is shown in Table 5.

TABLE 5. COMPOSITION OF UNESTERIFIED FATTY ACIDS FROM RAT CEREBRAL CORTEX

PROBABLE STRUCTURE	% COMPOSITION
14:0	1.57 ± 0.13
15:0	1.32 ± 0.17
16:1	1.90 ± 0.20
16:0	18.69± 0.63
* 17:1	6.21 ± 0.41
18:1/18:2	15.58 ± 0.28
18:0	19.15 ± 0.75
Unknown	4.76 ± 0.23
Unknown	4.16 ± 0.49
20:4	26.57 ± 1.12

Results are expressed as percentage composition by weight, and are given as mean ± S.E. of 10 experiments

The major fatty acids isolated were palmitic, oleic, linoleic, stearic and arachidonic acids. In addition there were present two unknown fatty acids, both were found to occur frequently and in considerable amounts. The positions of the methyl esters of the two unknown fatty acids, designated C_1 and C_2, relative to the known methyl esters may be seen from the chromatogram trace reproduced in Fig. 4.

* C17:1 was identified by comparison with the fatty acid retention times reported by Lunt (1967). The two unknown fatty acids correspond to the two fatty acids C19:0 and C19:1 (Lunt, 1967).

Fig. 4 Unesterified fatty acids from rat cerebral cortex

The trace shown is of a sample of unesterified fatty acids from rat cerebral cortex. The acids were isolated by the solvent extraction procedure. Methyl esters of the isolated fatty acids were chromatographed on Apiezon L grease at 200°C

A methyl dodecanoate

B methyl tridecanoate

C methyl tetradecanoate

D methyl pentadecanoate

E methyl hexadecamonoenoate

F methyl palmitate

G methyl heptadecamonoenoate

H methyl heptadecanoate - internal standard (10 µg)

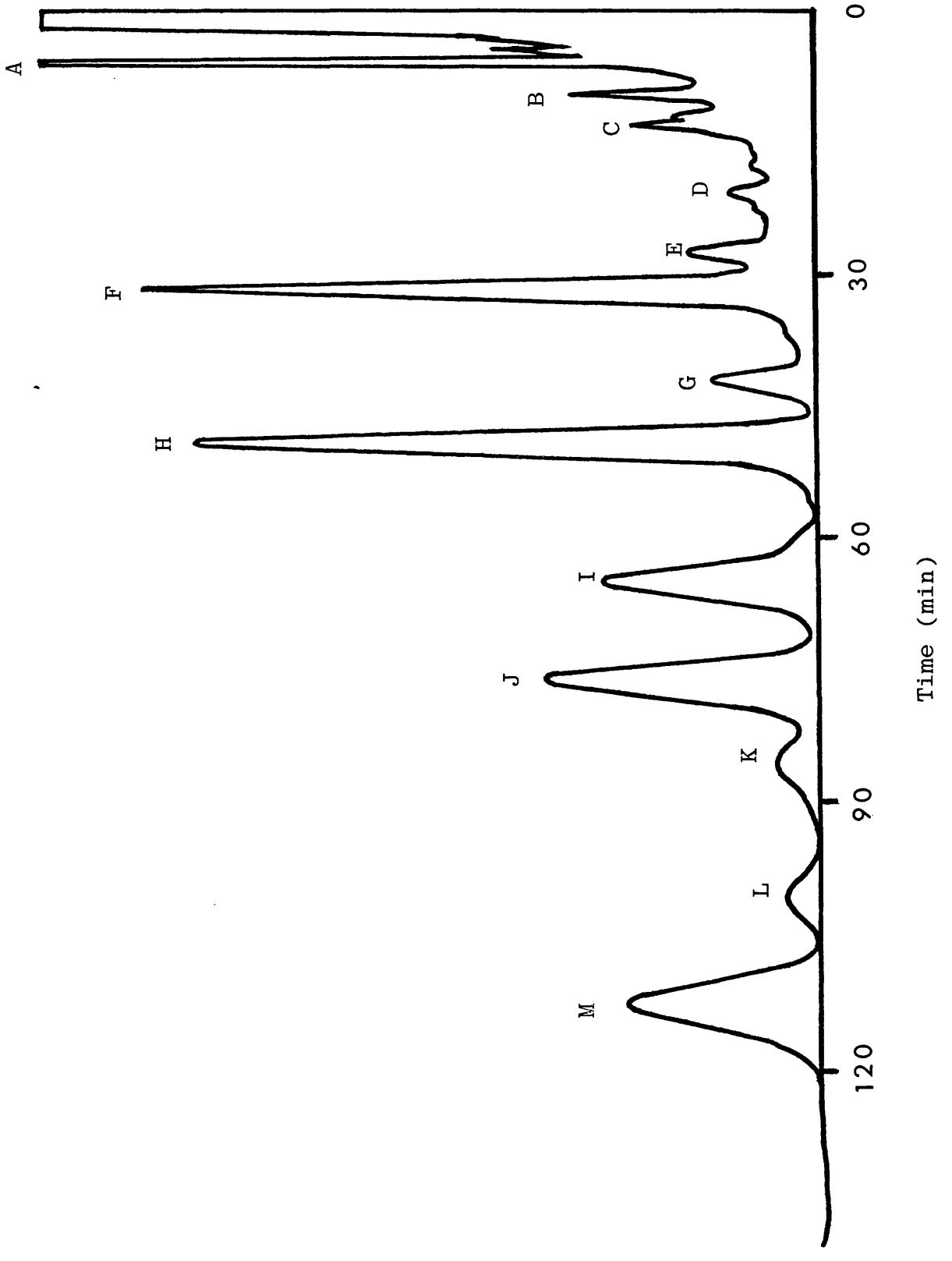
I methyl oleate and linoleate

J methyl stearate

K unknown C_1

L unknown C_2

M methyl arachidonate

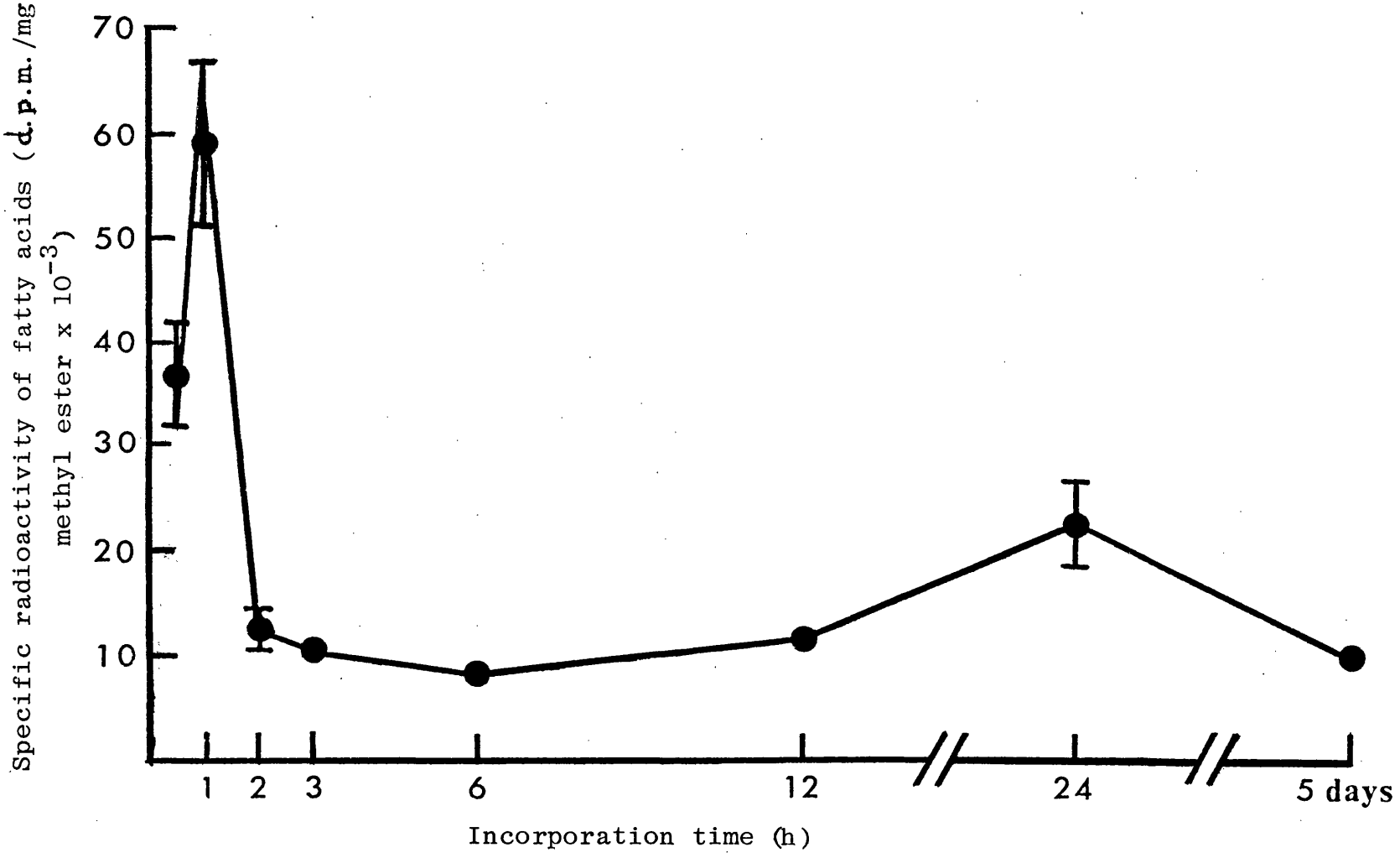

A
B
C
D
E
F
G
H
I
J
K
L
M
0
30
60
90
120
Time (min)

(ii) The incorporation of [1 - ^{14}C] acetate into the unesterified fatty acids of rat cerebral cortex *in vivo*

A series of preliminary experiments was done to establish the time course of incorporation of [1 - ^{14}C]acetate into the unesterified fatty acids of rat cerebral cortex *in vivo*. Rats received 2μCi of [1 - ^{14}C] acetate intraventricularly and were killed at varying time intervals after injection. The cerebral cortex was removed and the lipids extracted by homogenising in chlorofom-methanol (2:1, v/v). Unesterified fatty acids were isolated using the thin-layer chromatographic procedure described in Methods 4(i), and the specific radioactivity determined. The results of this preliminary investigation are shown in Fig. 5. Each point represents the mean ± S.E. (vertical lines) of three separate experiments. The results indicate that there is a rapid incorporation of acetate into the unesterified fatty acids of rat cerebral cortex *in vivo*, and that the acids appear to turnover rapidly.

These preliminary experiments indicated that at short incorporation times a considerable amount of unmetabolised [1 - ^{14}C]acetate was present in the excised cerebral cortex, which resulted in contamination of the isolated unesterified fatty acid fraction.

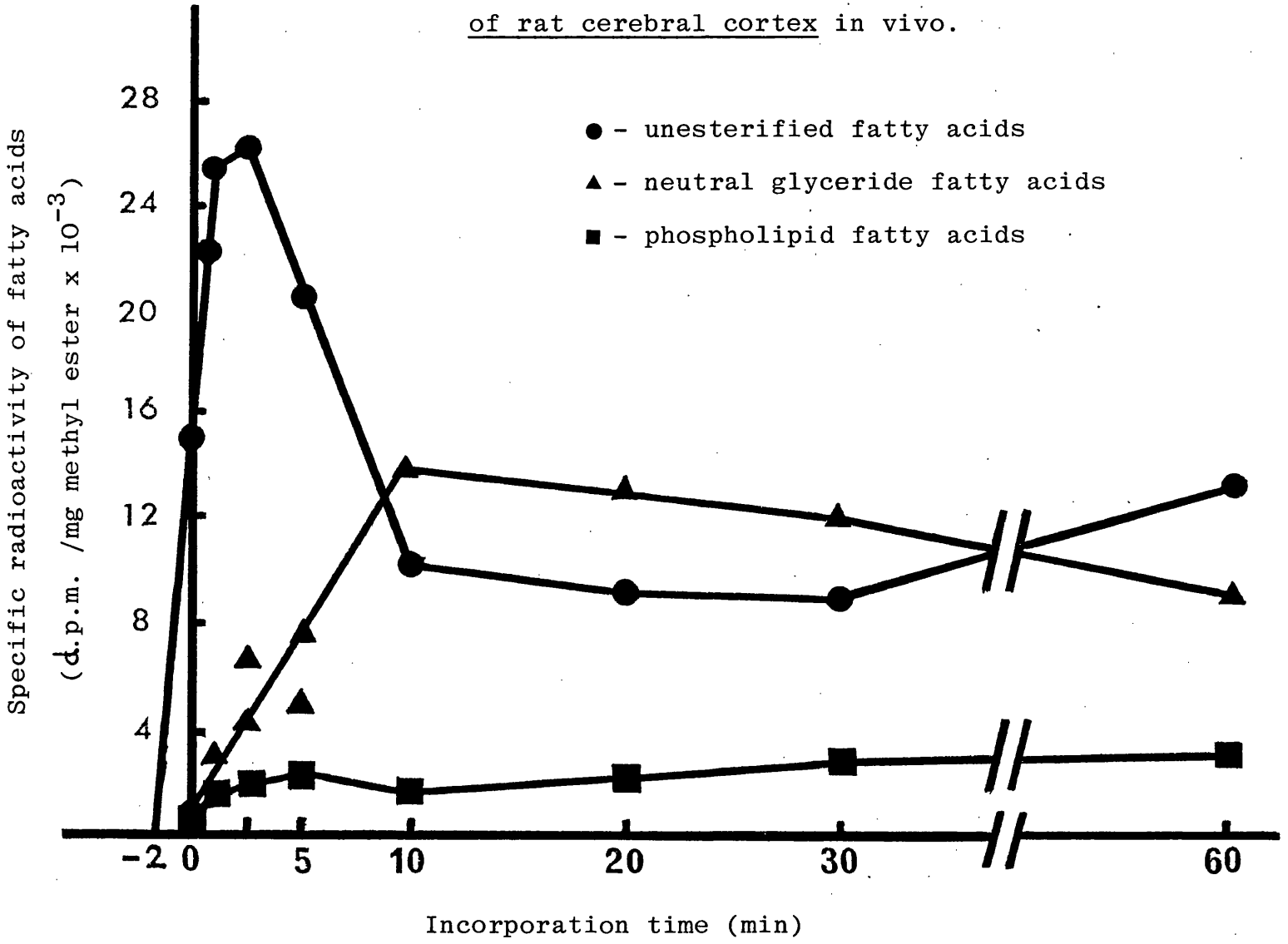
Fig. 5. The incorporation of 1 - ^{14}C acetate into the unesterified fatty acids of rat cerebral cortex in vivo.



A further set of experiments was carried out to determine the time-course of incorporation of [1 - ^{14}C] acetate into the three main classes of fatty acid in rat cerebral cortex *in vivo* and particular emphasis was given to short incorporation times (0-60 min). Rats received 2 μCi of [1 - ^{14}C] acetate by intraventricular injection and were killed at varying time intervals. The cortex was rapidly removed, homogenised in barbital-acetate buffer and washed to remove unmetabolized [1 - ^{14}C] acetate, using the procedures described in Methods 8 (i) and (ii). Unesterified fatty acids and the fatty acids of neutral glycerides and of phospholipids were isolated and their specific radioactivities determined. Unesterified fatty acids were separated from neutral glycerides using the solvent extraction technique described in Methods 4 (ii). The time between decapitation and homogenisation of the excised cortex was 2.0 ± 0.25 min (40 determinations). The pattern of incorporation of acetate into the fatty acid fractions is shown in Fig. 6. Each point is the mean of four separate experiments. During the 2-min period between decapitation and homogenisation acetate incorporation could proceed rapidly, therefore the true 'zero incorporation time' can be obtained only be extrapolation and is represented by the -2.0 min value in Fig. 6.

The percentage of the total radioactivity present in each fatty acid fraction attributable to unmetabolized [1 - ^{14}C]

Fig. 6 The incorporation of 1 - ^{14}C acetate into the fatty acids of rat cerebral cortex in vivo.



acetate is presented in Table 6. The values are calculated from the data in Table 3 and Fig. 6 using the levels of incorporation seen at 0 min, which represents a true incorporation period of 2.0 min. All values are given as the mean ± S.E. (no. of experiments).

TABLE 6. [1 - ^{14}C] ACETATE IN FATTY ACID FRACTIONS FROM RAT CEREBRAL CORTEX LIPIDS

FRACTION	% OF RADIOACTIVITY RECOVERED AFTER INJECTION ATTRIBUTABLE TO UNMETABOLIZED ACETATE
Initial tissue homogenate	-
Unesterified fatty acids	7.5 ± 1.1 (4)
Neutral glyceride fatty acids	0.9 ± 0.1 (4)
Phospholipid fatty acids	3.6 ± 0.4 (4)

(iii) The incorporation of [1 - ^{14}C] acetate into the unesterified fatty acids of rat cerebral cortex subcellular fractions *in vivo*.

Experiments were done to establish the incorporation of radioactively labelled acetate into unesterified fatty acids from sub-cellular fractions of rat cerebral cortex *in vivo*.

A total of twelve rats were used for the determination and the cerebral cortices from two rats were pooled for each experiment.

Rats received 2 μCi of [1 - ^{14}C] acetate as described previously and were killed by decapitation after 2.5 min. The cerebral cortex was substantially freed from white matter and fractionated according to the scheme outlined in Fig. 2. The synaptic membrane fractions were combined, pelleted and then extracted with chloroform-methanol as described in Methods 2. The myelin fraction was also removed, pelleted and extracted with chloroform-methanol. Solvent was removed from the total lipid extract by rotary evaporation and the dry lipid film treated with diazomethane as described in Methods 7. Unesterified fatty acid methyl esters were isolated by thin-layer chromatography on silica gel G using the solvent system light petroleum (b.p. 40-60^{o}C) - diethyl ether-acetic acid-(90:10:1, by vol.). The fatty acid methyl ester band was removed and eluted three times with 5 ml of diethyl ether. The specific radioactivities of the isolated fatty acid methyl esters are shown in Table 7.

TABLE 7. THE INCORPORATION OF [1 -^{14}C] ACETATE INTO THE UNESTERIFIED FATTY ACIDS ASSOCIATED WITH SUBCELLULAR FRACTIONS OF RAT CEREBRAL CORTEX *IN VIVO*.

FRACTION	YIELD OF METHYL ESTERS (μg)	TOTAL RADIOACTIVITY (d.p.m.)	SPECIFIC RADIOACTIVITY (d.p.m./mg methyl ester)
Synaptic Membranes	123.8±13.28(6)	998±101(6)	8266±963(6)
Myelin	424.0±52.45(6)	1533±98(6)	3858±428(6)

Results are expressed as mean± S.E. (no. of experiments)

Control experiments were performed to determine what percentage of the radioactivity present in the isolated fatty acid methyl ester fraction could be attributed to unmetabolized [1 - ^{14}C] acetate. Radioactively labelled acetate (4 μCi) was added to the cerebral cortex homogenates and the unesterified fatty acids associated with synaptic membranes and myelin isolated as described earlier. The results are shown in Table 8.

TABLE 8. [1 - ^{14}C] ACETATE IN THE UNESTERIFIED FATTY ACID FRACTIONS ASSOCIATED WITH SYNAPTIC MEMBRANES AND MYELIN

FRACTION	TOTAL RADIOACTIVITY OF FRACTION (d.p.m.)	% OF RADIOACTIVITY ATTRIBUTABLE TO UNMETABOLIZED ACETATE
Synaptic membranes	41.0 ± 6.2 (4)	4.35 ± 0.48 (6)
Myelin	22.8 ± 4.5 (4)	1.51 ± 0.1 (6)

All values are given as the mean ± S.E. (no. of experiments)

SECTION 3

LONG-CHAIN FATTY ACYL-CoA ESTER METABOLISM IN RAT BRAIN *IN VIVO*

(i) Level of long-chain fatty acyl-CoA esters in rat brain

Long-chain fatty acyl-CoA esters were extracted and measured by determining the CoA-SH liberated after alkaline hydrolysis, as described in Methods 9. The level of long-chain acyl-CoA esters in rat brain is 12.5 ± 0.42 nmoles/g wet weight (mean ± S.E. 14 determinations). The standard curve for the estimation of CoA-SH is shown in Fig. 7. The levels of long-chain acyl-CoA esters are expressed as nmoles/g wet weight. The tissue wet weights were calculated from the dry weight of the residue remaining after acyl-CoA extraction. The residue dry weight expressed as a percentage of the original wet weight of the tissue was 46.95% ± 1.05 (mean ± S.E. 7 determinations).

An experiment was done to estimate the recovery of the CoA-SH released by alkaline hydrolysis. Known amounts of authentic palmitoyl-CoA (range 2-5 nmoles) were hydrolysed and the CoA-SH released measured according to the procedures described in Methods 9 (ii) and (iii). The results are shown in Table 9.

Fig. 7. Standard curve for the estimation of CoA-SH

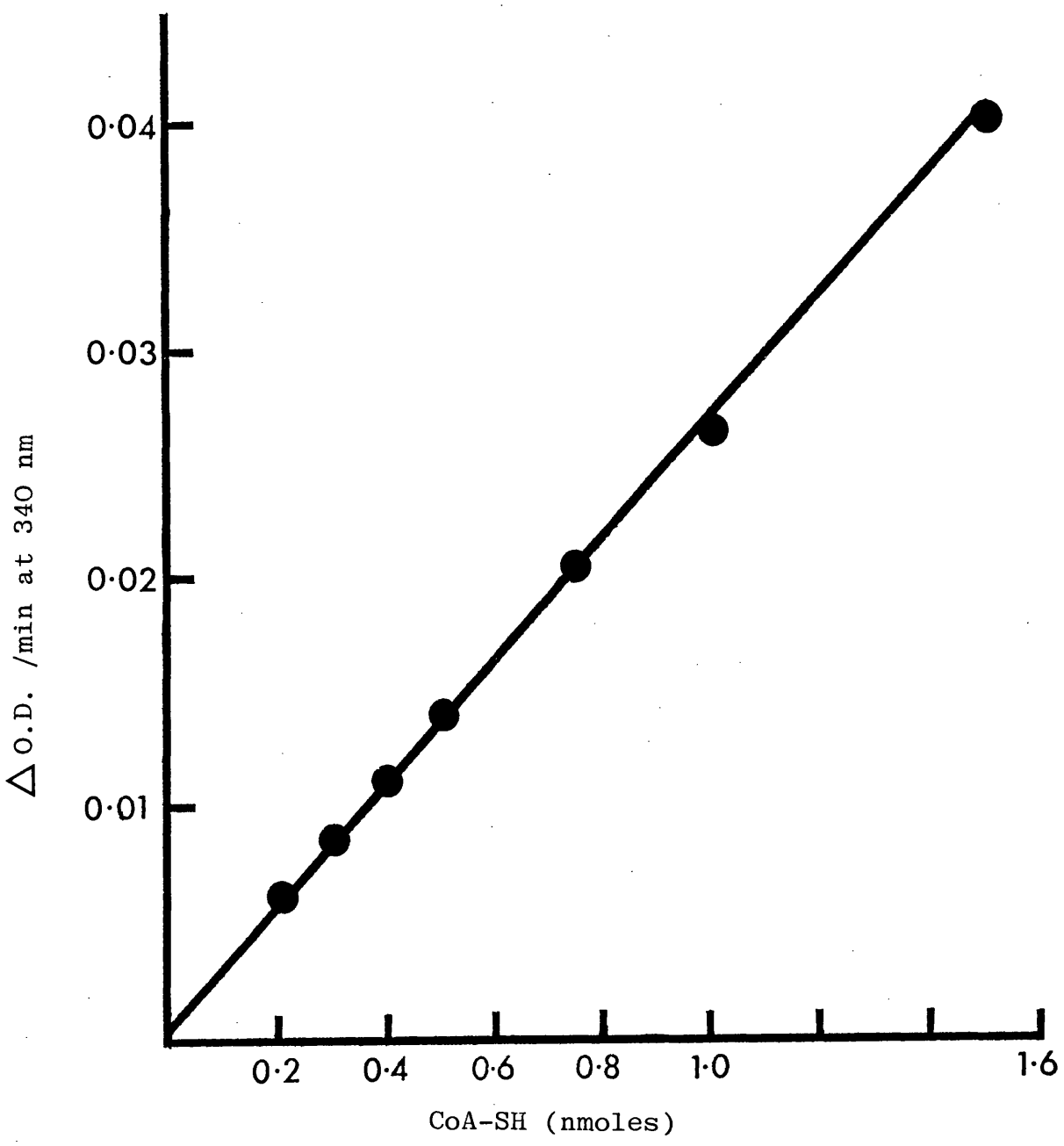


TABLE 9. RECOVERY OF CoA-SH RELEASED BY ALKALINE HYDROLYSIS OF ACYL-CoA ESTERS

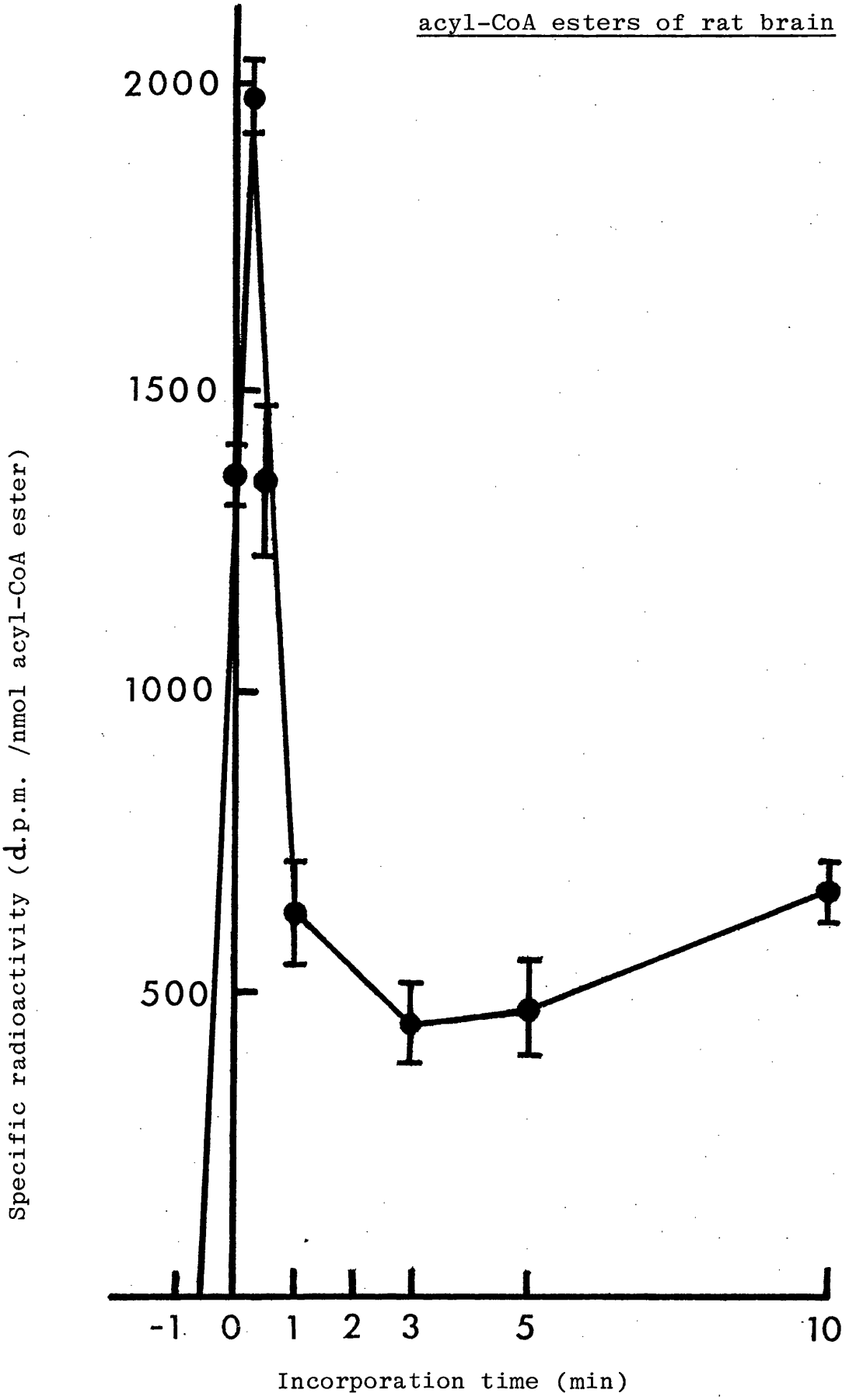
PALMITOYL-CoA HYDROLYSED (nmoles)	CoA-SH DETECTED (nmoles)	% RECOVERY
2.0	1.84	92
3.0	2.34	78
4.0	3.76	94
5.0	4.58	92

(ii) The incorporation of [1 - ^{14}C] acetate into the long-chain fatty acyl-CoA esters of rat brain *in vivo*.

A series of experiments was done to measure the incorporation of [1 - ^{14}C] acetate into the long-chain fatty acyl-CoA esters of rat brain *in vivo*. Rats received 2 µCi of [1 - ^{14}C] acetate by intraventricular injection and were killed by decapitation at varying time intervals. Long-chain acyl-CoA esters were isolated by the procedure described in Methods 10 (e), and were estimated by measuring the CoA-SH released following alkaline hydrolysis. The pattern of incorporation of acetate into acyl-CoA esters is shown in Fig. 8. Each point is the mean ± S.E. (vertical lines) of at least three separate experiments.

The yield of long-chain acyl-CoA esters using the isolation technique described in Methods 10 (e) was 3.03 ± 0.22 nmoles

Fig. 8 The incorporation of 1 - ^{14}C acetate into the long-chain fatty acyl-CoA esters of rat brain in vivo



(mean ± S.E. 22 determinations), which corresponds to a recovery of 25% ± 1.82 (mean ± S.E. 22 determinations) based on the level of acyl-CoA ester reported earlier.

Several experiments were performed in order to establish the amount of radioactivity present in the long-chain acyl-CoA ester fraction which was due to contaminating [1 - ^{14}C]acetate. Radioactively labelled acetate (2 μCi) was added to the chloroform-methanol homogenate and the long-chain fatty acyl-CoA esters isolated according to the procedure in Methods 10 (e). The radioactivity remaining in the acyl-CoA ester fraction is shown in Table 10.

TABLE 10. RECOVERY OF [1 - ^{14}C] ACETATE IN THE LONG-CHAIN FATTY ACYL-CoA FRACTION OF RAT BRAIN

FRACTION	TOTAL RADIOACTIVITY (d.p.m.)	% RADIOACTIVITY ATTRIBUTABLE TO UNMETABOLISED ACETATE*
Initial homogenate	4.4×10^6	-
Long-chain fatty acyl-CoA esters	542 ± 47.9 (6)	28 ± 1.6 (4)

* This value is calculated from the data in Fig. 8 using the level of incorporation seen at 1.0 min. Values are expressed as mean ± S.E. (no. of experiments).

SECTION 4

THE LONG-CHAIN FATTY ACYL-CoA HYDROLASE ACTIVITY OF RAT CEREBRAL CORTEX

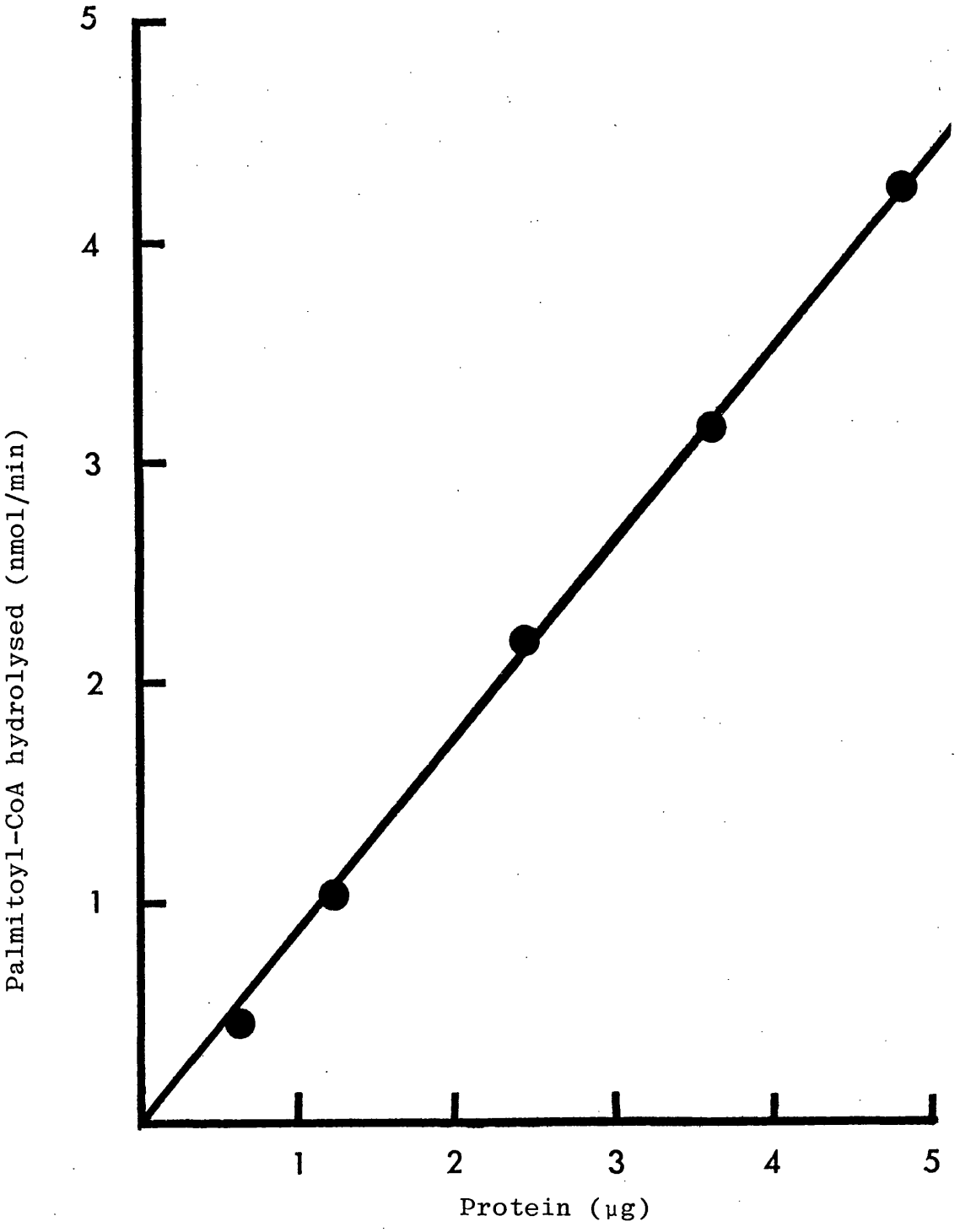
Experiments were performed in order to characterize some of the properties of long-chain fatty acyl-CoA hydrolase from rat cerebral cortex. In all the experiments hydrolase activity was measured in a high-speed supernatant from rat cerebral cortex, prepared by the procedure described in Methods 12.

Fig. 9 demonstrates that the rate of hydrolysis of palmitoyl-CoA is directly proportional to the amount of supernatant protein added, in the range 0 - 5 μg protein. The rate of hydrolysis was measured by the DTNB assay described in Methods 12 (i). Some of the properties of long-chain fatty acyl-CoA hydrolase from rat cerebral cortex are described below.

(i) Subcellular localization

Experiments were carried out to determine the subcellular localization of long-chain fatty acyl-CoA hydrolase activity in rat cerebral cortex. In each experiment the cerebral cortices from two rats were homogenised in 15 ml of ice-cold 0.32 M sucrose (dissolved in 50 mM phosphate buffer, pH 7) and the tissue fractionated according to the scheme outlined

Fig. 9. Rate of palmitoyl-CoA hydrolysis as a function of amount of high-speed supernatant protein.



in Methods 1 (ii). Each fraction was assayed for long-chain fatty acyl-CoA hydrolase activity using the DTNB assay procedure described in Methods 12 (i). The results are shown in Table 11.

TABLE 11. SUBCELLULAR LOCALIZATION OF LONG-CHAIN FATTY ACYL-CoA HYDROLASE ACTIVITY IN RAT CEREBRAL CORTEX

FRACTION	ISOLATION CONDITIONS	ENZYME UNITS / RAT CEREBRAL CORTEX EXPT.1	ENZYME UNITS / RAT CEREBRAL CORTEX EXPT.2	PERCENTAGE OF TOTAL UNITS EXPT.1	PERCENTAGE OF TOTAL UNITS EXPT.2
Total homogenate	10% Homogenate in 0.32 sucrose	32.73	35.08	100	100
Nuclear pellet	900 x g, 10 min 2 washings	6.49	6.12	19.8	17.4
Crude mitochondrial pellet	10,000 x g 20 min 1 washing	3.14	4.18	9.5	11.9
10,000 x g supernatant	10,000 x g 20 min	25.75	26.59	78.6	75.7
Overall recovery		35.38	36.89	108	105

The crude mitochondrial pellet was 'osmotically shocked' by homogenising in distilled water (see Fig. 2) and centrifuged at 100,000 x g for 1 h. The hydrolase activity present in the resulting supernatant and pellet is shown in Table 12.

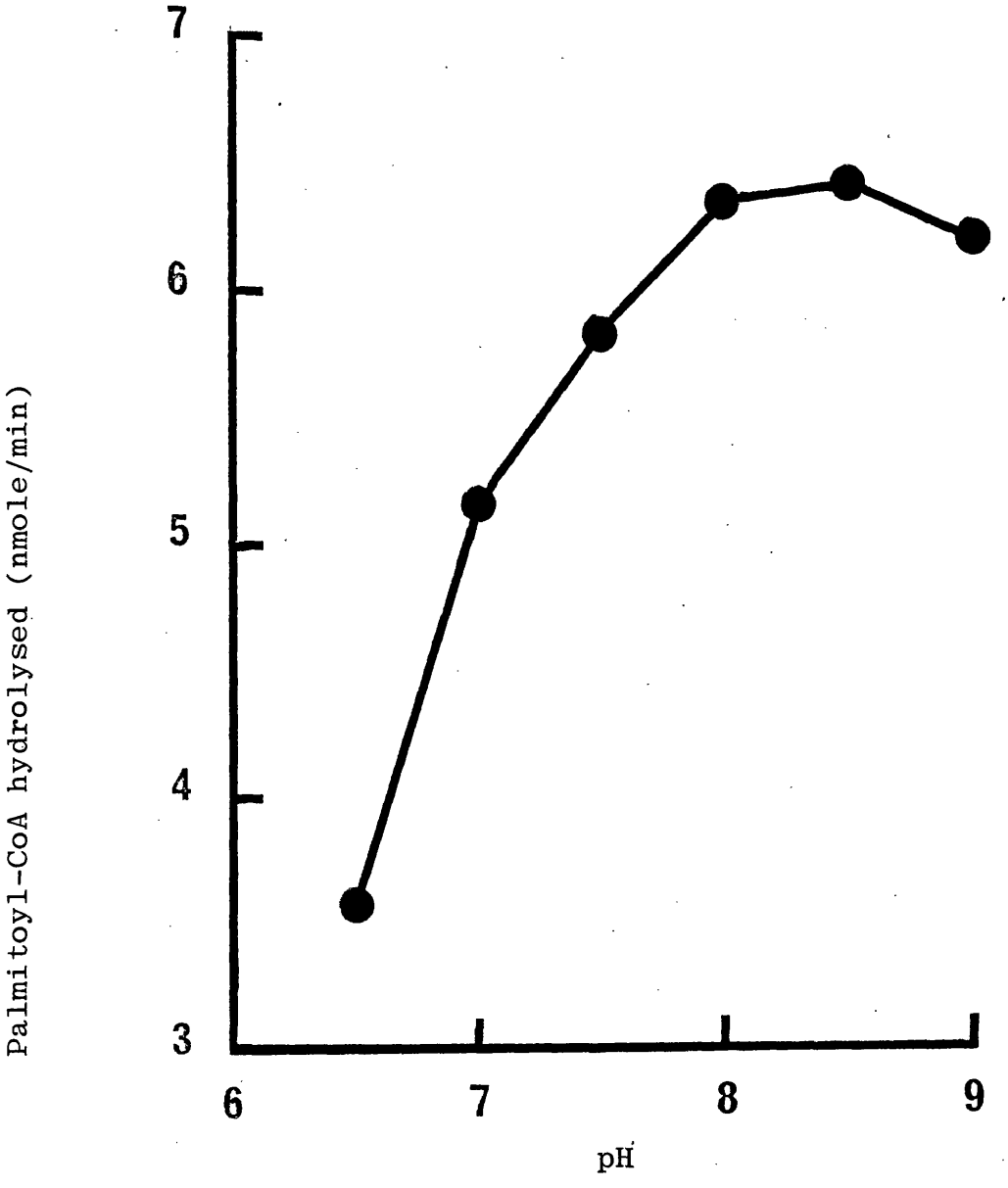
TABLE 12. DISTRIBUTION OF LONG-CHAIN FATTY ACYL-CoA HYDROLASE ACTIVITY IN THE CRUDE MITOCHONDRIAL PELLET FROM RAT CEREBRAL CORTEX

FRACTION	TOTAL ENZYME UNITS		PERCENTAGE OF TOTAL UNITS	
	EXPT.1	EXPT.2	EXPT. 1	EXPT.2
Crude mitochondrial pellet	3.14	4.18	100	100
Shocked pellet 100,000 x g supernatant	2.41	3.04	76.7	72.8
100,000 x g pellet	0.69	1.08	21.9	25.8
Recovery			98.7	98.6

(ii) Effect of pH on long-chain fatty acyl-CoA hydrolase activity.

The pH profile of long-chain fatty acyl-CoA hydrolase from rat cerebral cortex is shown in Fig. 10. The rate of hydrolysis of 40 nmoles of palmitoyl-CoA by 5 - 6 milliunits of

Fig. 10. Effect of pH on the hydrolysis of palmitoyl-CoA by long-chain fatty acyl-CoA hydrolase



enzyme was measured at various pH values using the DTNB assay procedure described in Methods 12 (i). The enzyme exhibited a broad pH profile with maximal activity between pH 8.0 and 8.5.

(iii) *Km* and *Vmax*

The initial rate of hydrolysis of palmitoyl-CoA and oleoyl-CoA was determined at different substrate concentrations using the DTNB assay described in Methods 12 (i). The results are expressed in the form of the 'direct linear plot' devised by Eisenthal and Cornish-Bowden (1974) and are shown in Figs. 11 and 12. The *Km* of the enzyme for palmitoyl-CoA and oleoyl-CoA was 1.4×10^{-6} M in each case. The *Vmax* using palmitoyl-CoA as a substrate was calculated to be 899 nmoles/min/mg protein and 265 nmoles/min/mg protein when oleoyl-CoA was used as a substrate.

(iv) Effects of calcium and magnesium on the activity of long-chain fatty acyl-CoA hydrolase.

The effects of Ca^{2+} and Mg^{2+} on the long-chain fatty acyl-CoA hydrolase activity of rat cerebral cortex are shown in Figs. 13 and 14. The hydrolase activity was assayed using a modification of the DTNB assay described in Methods 12 (i). Palmitoyl-CoA (5 nmoles) was added to a cuvette which contained 0.5 ml of the DTNB reagent and 0.5 ml of a solution of calcium or magnesium chloride. The mixture was incubated at $30^{o}C$ for

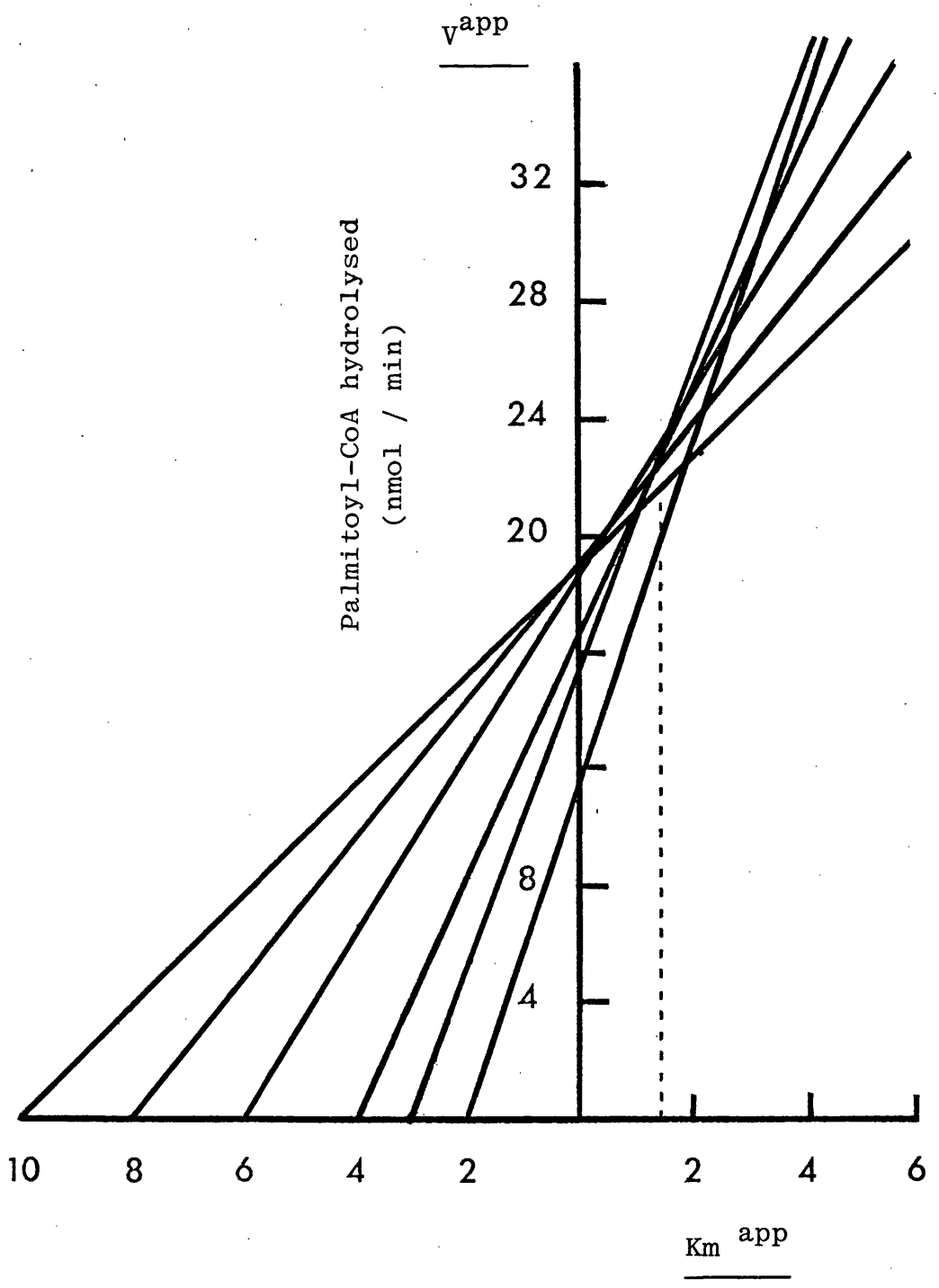


Fig. 11. Direct linear plot showing the kinetic parameters of long-chain fatty acyl-CoA hydrolase with palmitoyl-CoA as substrate.

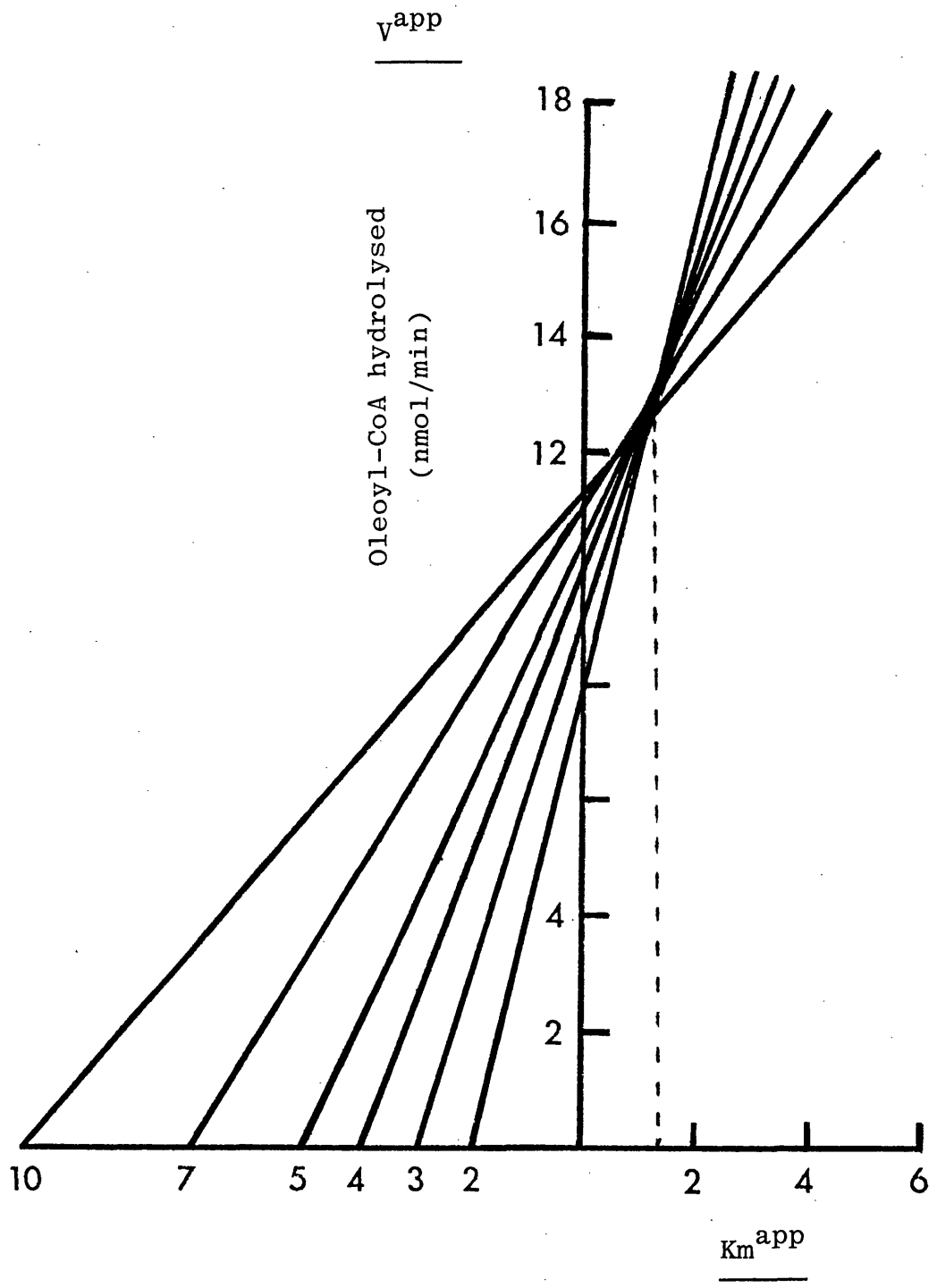


Fig. 12. Direct linear plot showing the kinetic parameters of long-chain fatty acyl-CoA hydrolase with oleoyl-CoA as substrate

5 min and the reaction started by adding approximately 1 milliunit of enzyme. The results demonstrate that the long-chain fatty acyl-CoA hydrolase is inhibited both by Ca^{2+} and Mg^{2+}. At 5 mM calcium chloride, hydrolase activity was reduced by 82% and at 5 mM magnesium chloride the hydrolase activity was reduced by 63%.

(v) Effect of bovine serum albumin on long-chain fatty acyl-CoA hydrolase.

The effect of bovine serum albumin on the long-chain fatty acyl-CoA hydrolase activity of rat cerebral cortex is shown in Fig. 15. The rate of hydrolysis of 5 nmoles of palmitoyl-CoA by approximately 1 milliunit of enzyme at various albumin concentrations was determined using the DCPIP assay described in Methods 12 (ii). The results demonostrate that the hydrolase activity is inhibited by bovine serum albumin, Inhibition was linear with albumin concentrations up to 1 mg/ml and could be reversed by the addition of excess substrate.

(vi) Effect of neurotransmitters

Long-chain fatty acyl-CoA activity remained unaffected by adrenaline, noradrenaline, 5-hydroxytryptamine, acetylcholine and carbachol at concentrations ranging from 10 to 100 μM. Hydrolase activity was measured by the DTNB assay.

Fig. 13. Effect of Ca^{2+} on the activity of long-chain fatty acyl-CoA hydrolase.

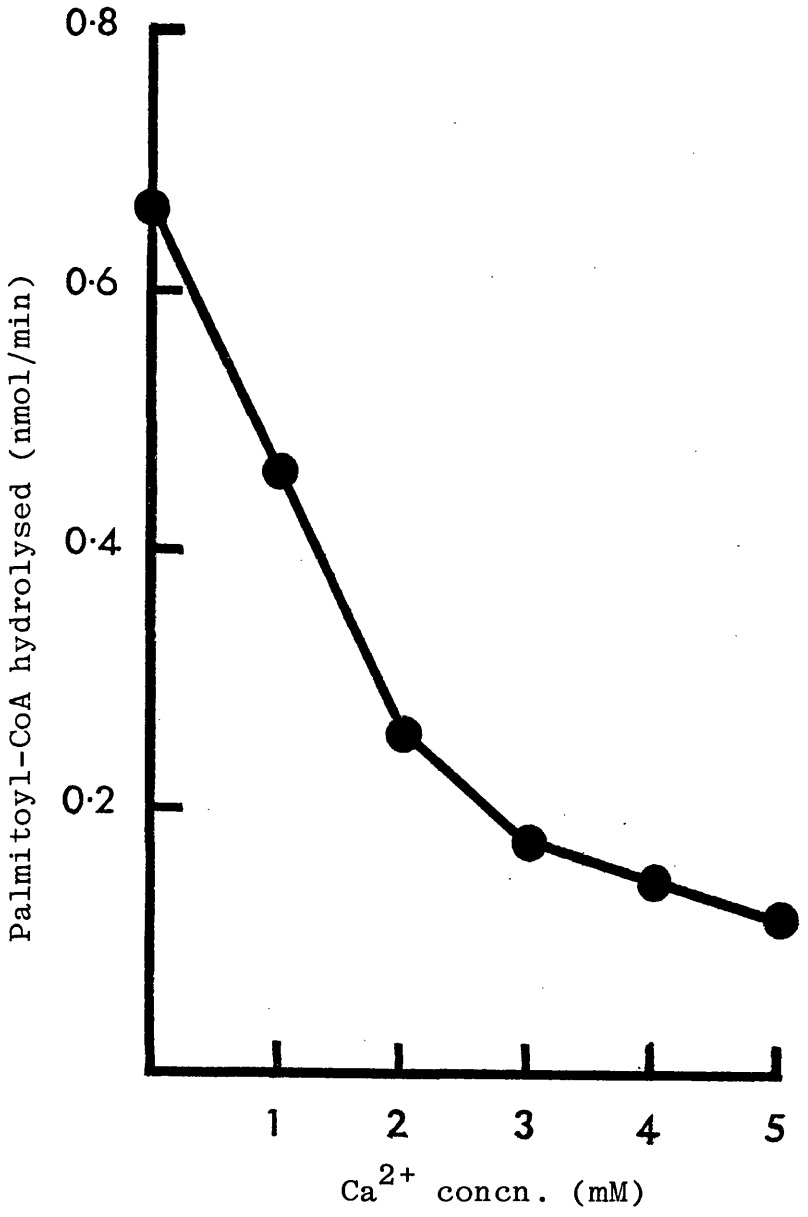


Fig. 14. Effect of Mg^{2+} on the activity of long-chain fatty acyl-CoA hydrolase.

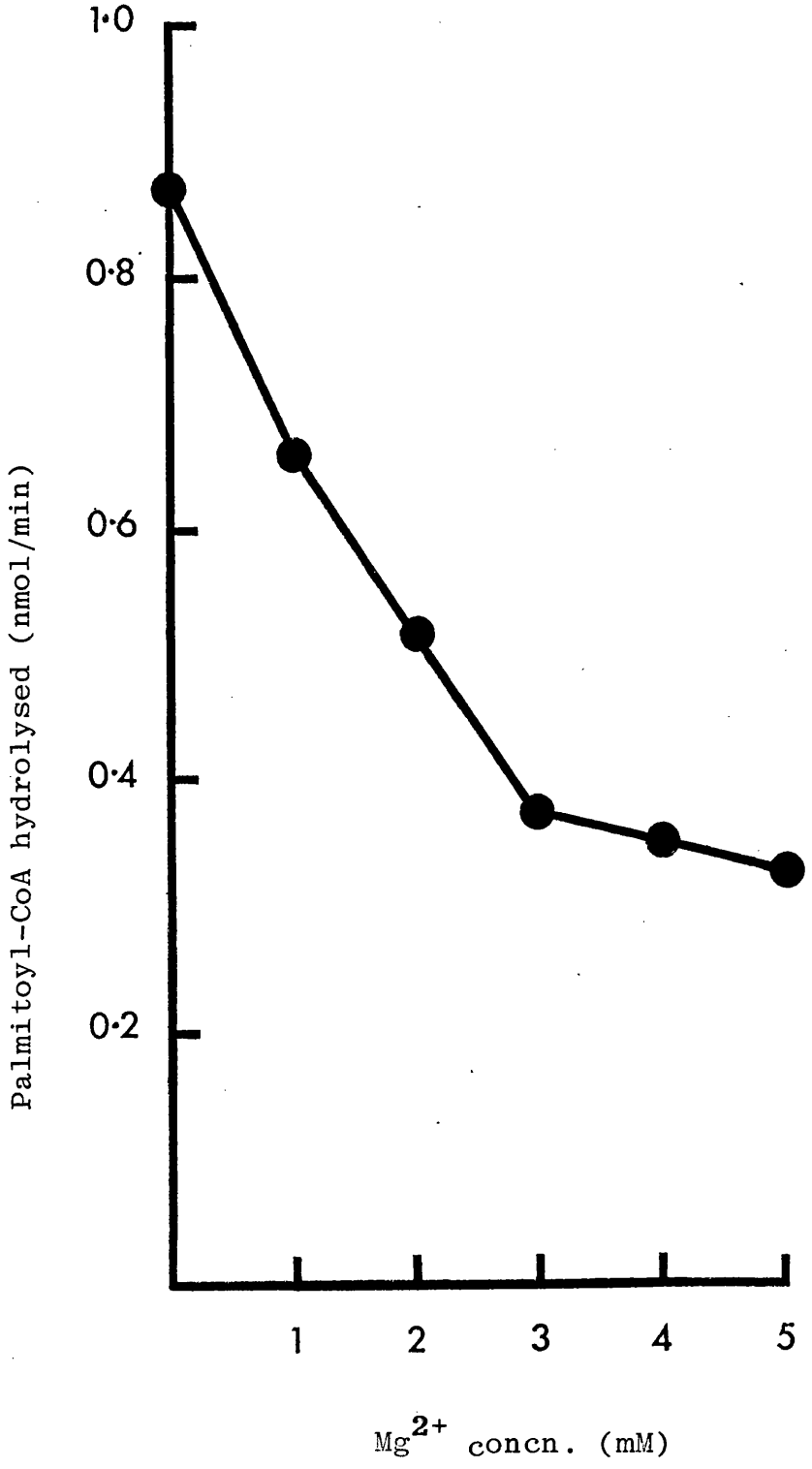
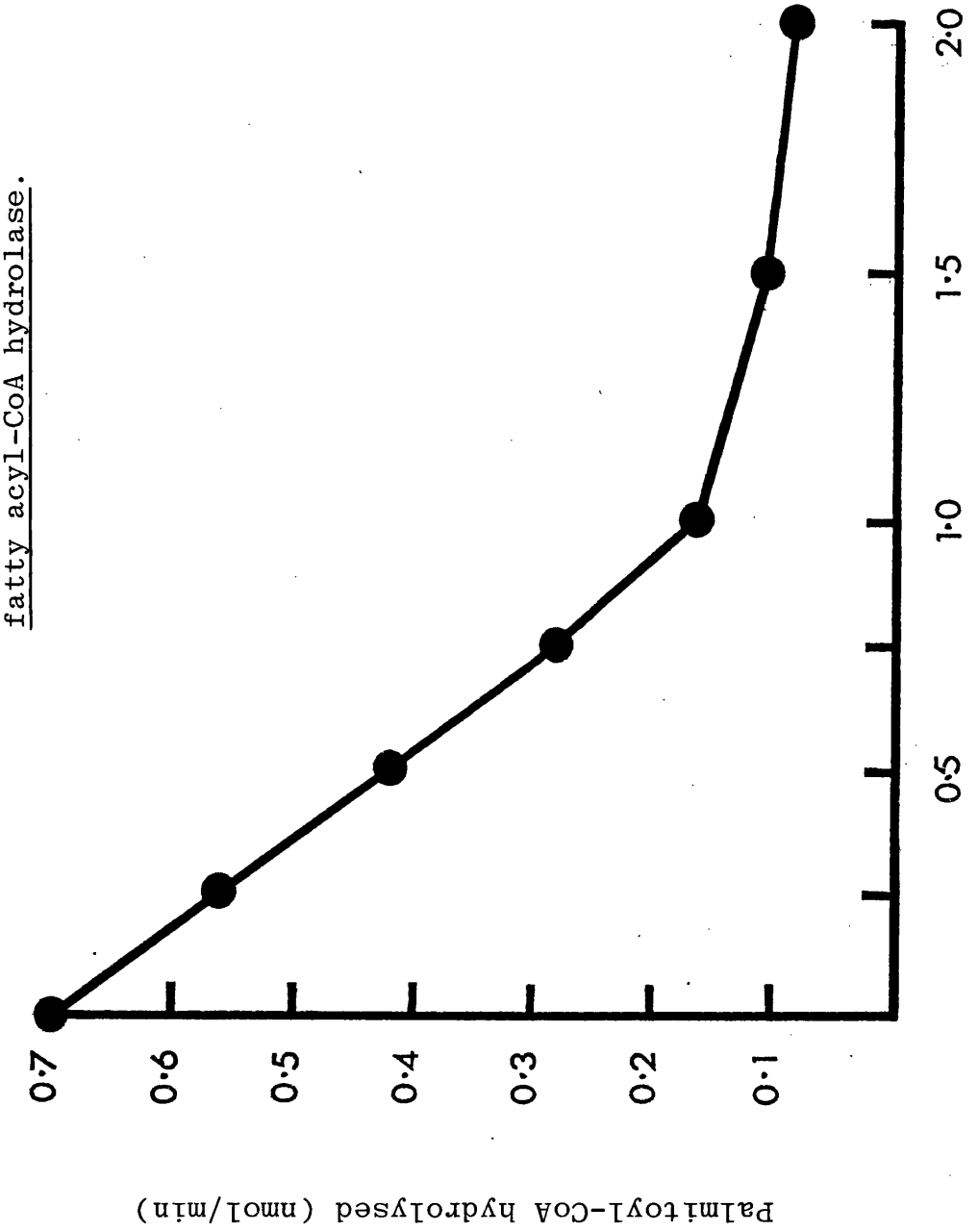


Fig. 15. Effect of albumin on the activity of long-chain fatty acyl-CoA hydrolase.



(vii)Effect of free fatty acid

The rate of hydrolysis of palmitoyl-CoA by long-chain fatty acyl-CoA hydrolase was not affected by the addition of palmitic acid at concentrations up to 100 μM. Hydrolase activity was measured by the DTNB assay.

DISCUSSION

1. DISCUSSION OF EXPERIMENTAL RESULTS

(i) THE LEVEL AND COMPOSITION OF UNESTERIFIED FATTY ACIDS IN RAT CEREBRAL CORTEX

The yield of unesterified fatty acid reported in Results section 2 corresponds approximately to 270 μg/g fresh tissue. This value is considerably higher than some of the levels reported by other workers (Bazan *et al.*, 1971; Cenedella, Galli and Paoletti, 1975). The time taken to excise the brain and prepare the cortex in the studies reported here was 2 minutes. The elevated level of unesterified fatty acid in the present study is in keeping with the observations of other workers that during the first 4 minutes of post-decapitation ischaemia in the rat there is a particularly rapid production of unesterified fatty acid (Bazan and Joel, 1968; Bazan *et al.*, 1971; Cenedella, Galli and Paoletti, 1975). The yield of unesterified fatty acid from cerebral cortex was variable, as is shown by the standard error for the reported value. A great deal of this variation may be attributable to differences in the time taken to excise the brain and dissect the cortex free from white matter, coupled with slight variations in the overall recovery of unesterified fatty acid.

The unesterified fatty acids isolated from rat cerebral cortex (See Table 5) are similar in composition to the acids isolated from whole brain by other workers (Lunt and Rowe, 1968;

Bazan, 1970; Cenedella, Galli and Paoletti, 1975). Palmitate, oleate, linoleate, stearate and arachidonate account for 80% of the total unesterified fatty acids. Arachidonic acid was by far the most abundant of all the acids, constituting 26% of the total. An elevated level of arachidonate is characteristic of brain which has been subjected to several minutes of post-decapitation ischaemia. Brain removed from rats within 30 seconds of decapitation has a considerably lower level of arachidonate (Bazan, 1970). Furthermore in brain removed from rats killed by immersion in liquid nitrogen arachidonate is totally absent (Lunt and Rowe, 1968). In view of these rapid changes in both the level and composition of the fatty acids, it is apparent that values obtained for cerebral cortex, rather than whole brain, may not be truly representative of the tissue *in vivo*.

(ii) THE METABOLIC ACTIVITY OF THE UNESTERIFIED FATTY ACIDS OF RAT CEREBRAL CORTEX *IN VIVO*

Examination of the data in Results section 1 shows clearly that the most efficient method for removing unmetabolized [1 -^{14}C] acetate is a combination of the two washing procedures described in Methods 8(i) and (ii). The washing procedures reduce the amount of [1 - ^{14}C] acetate contamination of the fatty acid fractions to an acceptable level.* The rapidly increasing radioactivity of the fatty acid fractions in Fig. 6 can therefore be considered to be indicative of metabolic incorporation of

* The author considers an acceptable level of contamination to be not greater than 10%.

acetate, and not simply contamination of the fractions by unmetabolized acetate. The level of incorporation into unesterified fatty acids seen at 0 min in Fig. 6 is considerably higher than could be accounted for by contamination from unmetabolized [1 - ^{14}C] acetate (see Table 6) indicating that incorporation proceeds rapidly during the 2 minute period between decapitation and homogenisation of the tissue.

The radioactive incorporation studies indicate that the unesterified fatty acids of rat cerebral cortex are metabolically very active. The results show there is a rapid incorporation of [1 - ^{14}C] acetate into the unesterified fatty acids of brain *in vivo* which supports previous observations made both *in vitro* and *in vivo* (Rowe, 1964; Lunt and Rowe, 1968; Dhopeshwarkar *et al.*, 1969a, 1971c; Carey, 1975a). The incorporation of acetate is primarily into palmitate (Rowe, 1964; Carey, 1975a) and much of the evidence suggests this is by synthesis *de novo* rather than by chain elongation of myristate (Dhopeshwarkar *et al.*, 1969a, 1971c). The unesterified fatty acids of rat cerebral cortex turn over rapidly *in vivo*, with a half-life of approximately 5 - 6 minutes. This value compares favourably with the turnover time of approximately 5 minutes for intracerebrally-injected oleic and arachidonic acids in mouse brain reported by Yau and Sun (1973, 1975). The time course of the appearance of labelled acetate in the neutral glyceride fraction also agrees well with the incorporation pattern of exogenous palmitic, oleic and arachidonic acids into di- and

triacylglycerols, in mouse brain *in vivo* (Sun and Horrocks, 1971; Yau and Sun, 1973, 1974). The appearance of labelled acetate in the total phospholipids of rat cerebral cortex also corresponds in some respects with the incorporation pattern of exogenous labelled fatty acids into individual phospholipids of mouse brain *in vivo* (Sun and Horrocks, 1971; Yau and Sun, 1973, 1974).

The specific radioactivity of the total phospholipid fatty acids was considerably lower than that of unesterified fatty acids and the fatty acids from neutral glycerides. This could be attributed to major differences in the metabolic activity of the individual phospholipids. Studies with labelled fatty acids have shown that phosphatidylinositol is rapidly labelled and has a much higher specific radioactivity compared to other phospholipids (Sun and Horrocks, 1971; Yau and Sun, 1973). However, phosphatidylinositol constitutes only 3% of the total brain phospholipids (Ansell, Dawson and Hawthorne, 1973). Thus the low phospholipid specific radioactivity reported in this work may, in part, be due to the dilution of the more highly labelled phospholipids by phospholipids with much lower specific radioactivities.

The incorporation of labelled acetate into the fatty acids of neutral glycerides and phospholipids must presumably occur through the esterification of pre-labelled fatty acids. The time course of the appearance of labelled acetate in the fatty acids of neutral glycerides, which agrees well with the reported incorporation

pattern of exogenous fatty acids, supports such a view. In addition the appearance of radioactivity in the fatty acids of neutral glycerides and phospholipids is marked by a fall in the specific radioactivity of the unesterified fatty acid fraction. There now appears to be ample evidence to suggest that, under normal, non-stimulated conditions the unesterified fatty acids of rat cerebral cortex are precursors for phospholipid and neutral glyceride biosynthesis *in vivo*.

The pattern of incorporation in Fig. 6 shows clearly that after 10 minutes the specific radioactivity of the unesterified fatty acid fraction shows only a slight variation. The preliminary experiments (see Fig. 5) showed little change in the specific radioactivity of the fatty acids from 2 hours to 5 days after incorporation of acetate. In both series of experiments the apparent lack of turnover may be partly explained by the recycling of the labelled fatty acids from the neutral glyceride and phospholipid fractions back into the unesterified fatty acid pool. A similar situation has been observed by Sun and Horrocks (1969) who used labelled palmitate to study the turnover of phospholipids in mouse brain *in vivo*. These workers found that the observed half-life of the phospholipids increased with increasing incorporation time.

The elevated level of unesterified fatty acid reported in this work, characterised by a higher percentage of arachidonate, is believed to be due to the release of esterified fatty acid following

decapitation (Bazan *et al.*, 1971). Such an increase in the unesterified fatty acid level after decapitation would be expected to lower the specific radioactivity of the acids. Bazan and co-workers (1971) reported that the level of unesterified fatty acid rises by 12 μmol/g fresh tissue/h. Therefore during the 2 minute period between decapitation and homogenisation the unesterified fatty acid level could have risen by as much as 0.4 μmol/g fresh tissue, effectively halving the specific radioactivity values. However there is evidence to suggest that the incorporation of acetate into unesterified fatty acids proceeds rapidly during the first 2 minutes of post-decapitation ischaemia. Thus the dilution effect from released fatty acids may be countered to some degree by the continued incorporation of acetate. The yield of unesterified fatty acid from the acetate incorporation experiments was 9.6 ± 0.35 μmol/g protein, which suggests that the release of the acids was reasonably constant. Although the observed specific radioactivity values are reduced during the 2 minute post-decapitation period they can be considered to reflect the pattern of incorporation of $[1 - {}^{14}C]$ acetate into unesterified fatty acids *in vivo*.

It has been shown recently that there is a rapid increase in the level of brain diglycerides during post-decapitation ischaemia (Banschbach and Geison, 1974; Aveldano and Bazan, 1975). The rapid release of diglyceride would be expected to lower the specific radioactivity of the neutral glyceride fraction,

in a manner similar to that previously described for the unesterified fatty acid fraction. The release of diglycerides rich in stearate and arachidonate suggests that they are derived from phospholipids by the action of a phospholipase C enzyme (Banschbach and Geison, 1974; Aveldano and Bazan, 1975). The release of diglycerides from phospholipids as a result of a non-specific phospholipase C would not be expected to have any gross effects on the specific radioactivity of the phospholipid fatty acid fraction. However Aveldano and Bazan (1975)have suggested that the release of diglycerides from phospholipids may be due to the action of a phosphatidylinositol-specific phospholipase C. The rapid incorporation of labelled fatty acids into phosphatidylinositol, and its high specific radioactivity compared to other phospholipids has been described earlier. A phospholipase C specific for the highly labelled phosphatidylinositol could significantly lower the specific radioactivity of the total phospholipid fatty acid fraction. Further information is needed on the specific radioactivity of individual phospholipids of cerebral cortex *in vivo*, using labelled acetate as the precursor and on the source of the released diglyceride, before such a view can be upheld. Until such evidence is forthcoming the incorporation of acetate into the neutral glycerides and the total phospholipids of rat cerebral cortex *in vivo* must be assumed to follow the pattern outlined in Fig. 6.

Preliminary experiments indicated that [1 - ^{14}C] acetate was rapidly incorporated into the unesterified fatty acids associated

with subcellular fractions of rat cerebral cortex *in vivo*. The fatty acids from the synaptic membrane fractions had a higher specific radioactivity than those associated with the myelin fraction. Lunt and Rowe (1968) have reported that acetate is incorporated into the unesterified fatty acids of subcellular fractions of rat brain *in vitro* and found that the acids associated with a combined synaptosomes and myelin fraction had a higher specific radioactivity than those from the microsomal, mitochondrial or supernatant fractions. Fractionation of brain increases the level of unesterified fatty acid and it is not known whether this increase is associated with any particular subcellular fraction (Lunt and Rowe, 1968). The increase may be due to the hydrolysis of membrane lipids during fractionation and the presence of phospholipase A1 and A2 in the subcellular fractions of cerebral cortex (Bazan, 1971c) suggests they may arise as a result of phospholipid degradation. Therefore the observed incorporation of acetate into the unesterified fatty acids of rat cerebral cortex subcellular fractions, could be due either to the labelling of separate pools of fatty acid or to the release of labelled esterified fatty acid.

(iii) LONG-CHAIN FATTY ACYL-CoA ESTER METABOLISM IN RAT CEREBRAL CORTEX *IN VIVO*

Long-chain acyl-CoA esters were estimated by measuring the CoA-SH released from the perchloric acid precipitate, by alkaline hydrolysis. Free CoA-SH, soluble acyl derivatives (acetyl-CoA,

succinyl-CoA, etc.) and disulphides, such as the mixed disulphides of coenzyme A and glutathione, are soluble in perchloric acid and are removed in the acid washes (Tubbs and Garland, 1964). The long-chain acyl-CoA esters are precipitated together with any short-chain acyl-CoA esters in the tissue pellet. Therefore the estimate of long-chain acyl-CoA esters reported in this work represents the yield of perchloric acid insoluble coenzyme A derivatives. The composition of the acid insoluble coenzyme A is not known, but it is considered to be composed essentially of long-chain fatty acyl-CoA esters (Tubbs and Garland, 1964; Denton and Halperin, 1968; Lee and Fritz, 1972).

Several experiments were performed in order to estimate the recovery of long-chain acyl-CoA esters, using palmitoyl - 1 - ^{14}C coenzyme A. The results indicated that the recovery of long-chain acyl-CoA esters in the tissue pellet is of the order of 95%. However the results proved to be misleading due to the fact that any radioactively labelled palmitic acid which may have been released during the isolation procedure was also recovered in the tissue pellet.

Examination of Fig. 8 indicates that [1 - ^{14}C] acetate is rapidly incorporated into the long-chain fatty acyl-CoA esters of rat brain *in vivo*. The percentage of the radioactivity of the acyl-CoA ester fraction which can be attributed to unmetabolized labelled acetate is high (see Table 10), however contamination by

[1 - ^{14}C] acetate cannot account for more than 30% of the radioactivity. The yield of long-chain acyl-CoA esters from the acetate incorporation study was variable and the recovery small. However the results from separate experiments suggest that the specific radioactivity values are comparable. Therefore the rapidly increasing radioactivity of the long-chain fatty acyl-CoA ester fraction can be considered to be indicative of metabolic incorporation of acetate. The level of incorporation into long-chain acyl-CoA esters seen at 0 min is much higher than could be accounted for by contamination from unmetabolized [1 - ^{14}C] acetate. This suggests that acetate is rapidly incorporated into acyl-CoA esters during the period between decapitation and homogenisation in chloroform-methanol. The time taken to remove the brains varied from 30 -40 seconds, therefore the true zero incorporation time is represented by the -0.5 min value in Fig. 8.

The long-chain fatty acyl-CoA esters of rat brain turn over rapidly *in vivo* with a half-life of approximately 30 seconds. A very short half-life would be consistent with the rapid rate of esterification of fatty acids into neutral glycerides and phospholipids reported earlier. The esterification of fatty acids into glycerides and phospholipids requires the activation of the acids to their corresponding acyl-CoA esters. Activation is accomplished by an enzyme termed an acyl-CoA synthetase and the presence of such an enzyme in brain has been reported by Cantrill and Carey (1975). The rapid incorporation of [1 - ^{14}C] acetate into long-chain fatty acyl-CoA esters can occur through two separate pathways. The first pathway involves the conversion of labelled fatty acids,

synthesised *de novo* from acetate, to long-chain acyl-CoA esters, by an acyl-CoA synthetase. The second pathway involves the chain elongation of existing acyl-CoA esters with radioactively labelled acetyl-CoA or malonyl-CoA. The presence of the two chain elongation systems in brain has been reported by Aeberhard *et al.* (1969). The rapid incorporation of labelled acetate into unesterified fatty acids is primarily into palmitate and is by synthesis *de novo* rather than chain elongation of myristate (Dhopeshwarkar *et al.*, 1969a, 1971c). Therefore at the very short incorporation times shown in Fig. 8 the appearance of radioactivity in the long-chain acyl-CoA ester fraction would presumably be due to the activation of palmitate synthesised *de novo* to palmitoyl-CoA. However the reappearance of label in the acyl-CoA esters after much longer incorporation times suggests that the chain elongation processes may also be contributing to the incorporation of acetate. The reappearance of radioactivity in the long-chain acyl-CoA ester fraction may also be due to the recycling of labelled palmitate. The radioactively labelled long-chain fatty acyl-CoA esters isolated in this work were not sufficiently pure to allow for a determination of the distribution and specific radioactivities of the fatty acids to be made.

(iv) THE LONG-CHAIN FATTY ACYL-CoA HYDROLASE ACTIVITY OF RAT CEREBRAL CORTEX

Long-chain fatty acyl-CoA hydrolase activity was measured in a 17,500 x g supernatant fraction of rat cerebral cortex. The results in Fig. 9 show that the rate of hydrolysis of palmitoyl-CoA is directly proportional to the amount of supernatant protein, when the substrate is in excess. These findings indicated that there were no factors, except the amount of enzyme, limiting the rate of reaction. The pH profile shown in Fig. 10 compares favourably with the profile reported by Kurooka *et al.* (1972) for the hydrolase activity of various rat organs. The hydrolase activity could not be determined at pH values above 9.0 due to the spontaneous hydrolysis of the substrate. Long-chain fatty acyl-CoA hydrolase from rat cerebral cortex hydrolysed palmitoyl-CoA and oleoyl-CoA with an apparent *Km* value of 1.4 μM for both substrates. The *Km* values reported by other workers for palmitoyl-CoA range from 3 - 5 μM (Anderson and Erwin, 1971; Kurooka *et al.*, 1972) and are above the critical micellar concentration for palmitoyl-CoA (3 - 4 μM) (Barnes, 1975). The *Km* reported in this work is below the critical micellar concentration and may be a truer reflection of the actual *Km* of the enzyme. The difference in the *Vmax* values with palmitoyl-CoA and oleoyl-CoA as substrates reflects the substrate specificity of long-chain acyl-CoA hydrolase. Kurooka *et al.* (1972) reported that the partially purified enzyme hydrolysed C_8 to C_{18} acyl-CoA esters, with myristoyl-CoA and

palmitoyl-CoA being the best substrates. Anderson and Erwin (1971) have shown that the enzyme from bovine brain can hydrolyse octanoyl-CoA and palmitoyl-CoA and that the longer chain thioester is the best substrate.

Brain has a higher long-chain fatty acyl-CoA hydrolase activity than any other tissue (Kurooka *et al.*, 1972). It is still possible however, to isolate long-chain fatty acyl-CoA esters, suggesting some sort of control or compartmentalization of the long-chain fatty acyl-CoA hydrolase *in vivo*. The subcellular localization is shown in Table 11. Between 75 and 80% of the total enzyme activity can be recovered in the 10,000 x g supernatant and approximately 10% is associated with the crude mitochondrial fraction. The results in Table 12 show clearly that the majority of the enzyme activity which is associated with osmotically sensitive structures is soluble in nature. The subcellular distribution of the hydrolase reported in this work compares favourably with the localization of the enzyme in bovine brain, described by Anderson and Erwin (1971). There now appears to be sufficient evidence to support the view that the long-chain fatty acyl-CoA hydrolase of brain is almost entirely soluble in nature, with a small percentage of the activity associated with the mitochondria. Acyl-CoA synthetase activity is associated essentially with the microsomal, mitochondrial and plasma membrane fractions of brain (Cantrill and Carey, 1975), thus it seems unlikely that compartmentalization is the mechanism for control of long-chain fatty acyl-CoA hydrolase activity in

rat cerebral cortex *in vivo*.

Long-chain fatty acyl-CoA hydrolase activity is inhibited both by Mg^{2+} and Ca^{2+} (see Figs. 13 and 14). Bivalent cations probably complex with the pyrophosphate group on the CoA molecule, producing a compound that is a poor substrate for the enzyme. The concentration of magnesium chloride and calcium chloride required for enzyme inhibition is high and it is doubtful whether such a control mechanism could exist *in vivo*. The effect of albumin on the hydrolase activity is shown in Fig. 15. Anderson and Erwin (1971) described a similar effect with the bovine brain hydrolase and suggested that inhibition was due to a binding of the acyl-CoA esters to the protein. The findings reported in this work support such a view since the inhibition can be reversed by adding excess substrate. The enzyme showed no substrate inhibition by released fatty acid. The long-chain fatty-acyl-CoA hydrolase of rat cerebral cortex showed no stimulation by neurotransmitters. However the possibility remains that control of the enzyme may be achieved in the intact cell via a cyclic nucleotide system such as has been reported by Gullis and Rowe (1975c) for the phospholipase A2 activity of guinea-pig brain. Until the effect of cyclic nucleotides on the long-chain fatty acyl-CoA hydrolase activity of rat cerebral cortex has been examined the most likely explanation for enzyme control *in vivo* is the binding of the acyl-CoA esters to a protein.

GENERAL DISCUSSION

Before this work was undertaken very little was known about the role of unesterified fatty acids in brain. It was clear that under stimulated conditions there was a rapid increase in the level of these acids (Bazan, 1970, 1971b; Bazan *et al.*, 1971; Lunt and Rowe, 1971), but their source was not firmly established. The relationship between unesterified fatty acids, phospholipids and acylglycerols was also unclear, therefore it was decided to investigate the relationship between the three main classes of fatty acid in brain *in vivo*. The early results indicated that acetate is rapidly incorporated into unesterified fatty acids and that they turn over very rapidly *in vivo*. It was observed that the acids were also very rapidly incorporated into acylglycerols and phospholipids under normal conditions *in vivo*. The esterification of fatty acids can only occur via their acyl-CoA esters, therefore the rate at which the acyl-CoA esters of brain turn over was also investigated. The presence of an acyl-CoA hydrolase in brain had been known for some time (Srere *et al.*, 1958; Vignais and Zabin, 1958) but nothing was known about the role of the enzyme in brain. The fact that long-chain fatty-acyl-CoA esters can be isolated from brain indicates that some sort of control mechanism for the enzyme must exist *in vivo*. A preliminary investigation of the control mechanism and role of the enzyme in brain was therefore made and the possibility that this enzyme might be involved in

maintaining the level of the unesterified fatty acid pool in brain was examined. The implications of this work are discussed below.

In the work presented in this thesis it has been demonstrated that under non-stimulated conditions *in vivo*, endogenous unesterified fatty acids are rapidly incorporated into phospholipids and neutral glycerides. Recently Sun and Yau (1976) have shown that exogenous oleic and arachidonic acids are incorporated into the membrane phospholipids of the synaptosomal-rich fraction of mouse brain *in vivo*.

Marked increases in the levels of unesterified fatty acids are seen under conditions of intense synaptic activity such as occur during electroshock treatment or drug-induced convulsions (Bazan, 1971b; Lunt and Rowe, 1971). It has been suggested that the release of these acids may be an important part of the mechanism for the regulation of synaptic membrane permeability (Lunt and Rowe, 1971; Bazan, 1971b). The possible sources of the released unesterified fatty acids have been described in the introduction to this work and it is suggested that the most likely one is the hydrolysis of membrane phospholipids. Gullis and Rowe (1975 a, c) have described a phospholipase A2 enzyme in guinea-pig synaptic membranes that is stimulated by neurotransmitters and by cyclic nucleotides. Under stimulated conditions increased phospholipase A2 activity could account for the rapid release of unesterified fatty acids. Phospholipase C activity coupled with di- and mono-acylglycerol lipase activities would

also release unesterified fatty acids from membrane phospholipids. Vyvoda and Rowe (1973) have reported the presence of neurotransmitter stimulated acylglycerol lipases in guinea-pig synaptic membranes but to date there is no evidence to suggest that any phospholipase C, other than the enzyme specific for phosphatidylinositol, is directly stimulated by neurotransmitters or cyclic nucleotides.

Gullis and Rowe (1975b) reported that under certain conditions the fatty acids released from synaptic membrane phospholipids, by the stimulated phospholipase A2, are rapidly taken up again. Further investigation revealed that transmitters which stimulated hydrolysis of membrane phospholipids under certain conditions also stimulated the acylation of endogenous lysophospholipids in synaptic membranes (Gullis and Rowe, 1975 b, c). Thus there is now good evidence that unesterified fatty acids are incorporated into the phospholipids of synaptic membranes under normal, non-stimulated conditions and that under conditions of intense synaptic activity unesterified fatty acids are released from membrane phospholipids by phospholipase action. This raises the question of the role of membrane phospholipid degradation and synthesis in the process of synaptic transmission and the part that unesterified fatty acids may play in this sequence of events.

The stimulated cycle of phosphatidylinositol degradation and resynthesis, by neurotransmitters, has been described in the

introduction to this thesis and there is much evidence to suggest that it is localized mainly in the pre-synaptic terminals rather than in the post-synaptic cells. Stimulated phosphatidylinositol breakdown, by phospholipase C, would account for the release of 1,2 diacylglycerols rich in stearate and arachidonate during post-decapitation ischaemia (Aveldano and Bazan, 1975). The mechanism by which this stimulation is evoked and the function of the stimulated phosphatidylinositol turnover is not yet clear. Pre-synaptic receptors, cyclic nucleotides and Ca^{2+} have been suggested as possible controlling factors (see Michell, 1975 for review). The indication that stimulated phosphatidylinositol turnover is mainly a pre-synaptic event suggests that it may be involved in the release of neurotransmitters. In experiments in which stimulation of turnover *in vivo* was examined the breakdown of phosphatidylinositol was most marked in fractions rich in synaptic vesicles and pre-synaptic plasma membranes (Lunt and Pickard, 1975). In addition the newly-synthesised phosphatidylinositol is also located mainly in these fractions (Yagihara *et al.* 1973; Schacht *et al.*, 1974). These observations have been interpreted as being indicative of a specific function for lipid turnover in synaptic vesicles and in the pre-synaptic terminal membranes, possibly related either to the interaction of synaptic vesicles and pre-synaptic plasma membranes during exocytosis or to the uptake of neurotransmitters into the vesicles (Yagihara *et al.*, 1973; Lunt and Pickard, 1975). Studies on the stimulated turnover of phosphatidylinositol suggest that only

the phosphorylinositol group undergoes renewal and that the diacylglycerol backbone of the molecule is reutilized during resynthesis (see Michell, 1975 for review). However as stated previously Aveldano and Bazan (1975) have observed the release of 1,2 diacylglycerols rich in arachidonate during post-decapitation ischaemia and suggest that these diglycerides arise from phosphatidylinositol. Release of free arachidonate is also a feature of the ischaemic brain and it seems likely that a major source of the free acid would be the arachidonate-enriched 1,2 diacylglycerol. Under ischaemic conditions therefore the acylglycerol lipases described by Vyvoda and Rowe (1973)may act on this diglyceride, whereas under normal conditions the diglyceride remains intact and is reutilized for phosphatidyl-inositol synthesis.

The stimulation of phospholipase A2 by exogenous neuro-transmitters suggests that deacylation may be a post-synaptic occurrence. Deacylation of membrane phospholipids would be expected to alter some of the properties of the membrane, e.g. permeability to cations, and this may be part of the mechanism for controlling the permeability of the post-synaptic membrane. Reacylation would then presumably be necessary for the recovery of the normal membrane permeability. The presence of a neuro-transmitter and cyclic nucleotide-stimulated phospholipase A2-acylation system in guinea-pig synaptic membranes (Gullis and Rowe (1975 a, b, c) supports the suggestion that a deacylation-reacylation cycle has a role in modulating post-synaptic membrane

permeability. The proposed system of control described by Gullis and Rowe (1975c), which is outlined in the Introduction, explains the stimulations of both deacylation and reacylation by neurotransmitters and cyclic nucleotides. The stimulated incorporation of oleate into synaptic membrane phospholipids (Gullis and Rowe, 1975 b, c) suggests that an acyl-CoA synthetase is present in synaptic membranes. It has been reported that the nuclear fraction from brain, which contains nuclei and cell membrane fragments, contains between 80 - 90% of the total acyl-CoA synthetase activity (Pande and Mead, 1968; Cantrill and Carey, 1975). The activity may be associated with glial cell plasma membranes as well as with synaptosomal membranes and the hypothesis has been advanced that a plasma membrane-bound acyl-CoA synthetase may be associated with the transport of fatty acids into the brain (Pande and Mead, 1968). The sub-cellular fractionation techniques available at the moment are unable to separate synaptic membranes from contaminating glial cell membranes. Therefore the precise nature and origin of the plasma membrane-bound acyl-CoA synthetase cannot at present be ascertained. Until such information is forthcoming it may be assumed that the incorporation of fatty acid into synaptic membrane phospholipids reported by Gullis and Rowe (1975 b, c) involves a synaptic-membrane-bound acyl-CoA synthetase. There is now good evidence that unesterified fatty acids can be incorporated into synaptic membrane phospholipids by a stimulated acylase system and the possibility that they are essential for

the changes in membrane permeability during the process of synaptic transmission still exists. It should be emphasised that very little is known about the rate of the enzymic reactions which are considered to be involved in these events. Furthermore, it is not yet known how much membrane phospholipid needs to be broken down before any changes in permeability occur.

Aveldano and Bazan (1974) have shown that when retina is incubated *in vitro*, highly unsaturated unesterified fatty acids, probably arising from plasma membrane lipids, rapidly accumulate in the incubation medium when albumin is present. After 20 minutes of incubation, most of the unesterified fatty acids double their concentration in the tissue, however the tissue concentration of some of the polyunsaturated fatty acids remains unchanged. Bazan and Bazan (1975) have reported that retina incubated under similar conditions incorporates ^{3}H-arachidonic acid into membrane phospholipids. These observations indicate that some of the polyunsaturated fatty acyl groups of retinal membrane phospholipids are continually being removed and replaced and it is suggested that the membrane may exist in a state of dynamic equilibrium. The incorporation of labelled exogenous oleic and arachidonic acids into synaptosomal membrane phospholipids *in vivo* (Sun and Yau, 1976) suggests that a similar situation may occur in synaptic membranes. Thus under non-stimulated conditions *in vivo* it could be envisaged that the synaptic membrane exists in a state of equilibrium and that the membrane phospholipids are rapidly deacylated and reacylated. The question which remains

unanswered is, could the enzymatic degradation and resynthesis of membrane phospholipids take place within the time scale known for the process of synaptic transmission? It seems unlikely that the whole sequence could be completed within the time of 1 - 2 milliseconds required for the generation of an action potential. However changes of the sort described would certainly alter the permeability of the post-synaptic membrane and as such may affect the response of the membrane to released neuro-transmitter. Thus it may be that unesterified fatty acid release and uptake is part of the mechanism of facilitation of synaptic transmission rather than having a direct role in the primary event of membrane depolarisation or hyperpolarisation that follows transmitter-receptor interaction.

The release of unesterified fatty acids could have effects on other cellular processes; they are potent uncouplers of oxidative phosphorylation (Björntorp *et al.*, 1964; Chefurka, 1966; Chefurka and Dumas, 1966); in low concentrations they affect certain key enzymes involved in glucose metabolism (Krebs *et al.*, 1965; Weber *et al.*, 1966; Williamson *et al.*, 1966); free arachidonic acid is a necessary precursor for prostaglandin biosynthesis (Lands and Samuelsson, 1968; Vonkeman and Van Dorp, 1968) and unesterified fatty acids inhibit Na-K-dependant ATP-ase in brain (Ahmed and Thomas, 1971). Thus it appears that a number of metabolic processes may be affected by or may be dependant upon the components of the unesterified fatty acid pool.

It has been suggested that the rapid release of unesterified fatty acids during post-decapitation ischaemia and electroshock treatment is due to the activation of a phospholipase A enzyme (Bazan, 1970). However this rapid release of acids could be simply an effect caused by the ischaemic or hypoxic condition of brain. The levels of phosphocreatine and ATP are lowered after electroshock treatment (Minard and Davis, 1962; King *et al.*, 1967) and following decapitation (Lowry *et al* ., 1964). If there is a rapid deacylation and reacylation of membrane phospholipids *in vivo* under normal conditions, as suggested previously, the rapid fall in ATP levels, observed during electroshock treatment, could result in a reduced rate of reacylation of membrane phospholipid and therefore if deacylation, a process which does not require ATP, continues the level of unesterified fatty acid in brain will rise. A similar argument can be advanced to explain the rapid production of 1,2 diacylglycerols rich in stearate and arachidonate during post-decapitation ischaemia. The resynthesis of phosphatidylinositol from diglyceride and inositol requires ATP and CTP. Reduced phosphatidylinositol resynthesis would result in the accumulation of 1, 2 diacylglycerols rich in stearate and arachidonate. Therefore the release of membrane lipids under conditions of 'intense synaptic activity' may not necessarily be compatible with the normal functioninng of the nervous system.

Long-chain fatty acyl-CoA esters

It has been shown that rat brain contains a pool of long-chain fatty acyl-CoA esters which is metabolically very active. The long-chain acyl-CoA esters turn over very rapidly *in vivo* and it is suggested that this is due to the esterification of the fatty acyl groups into neutral glycerides and phospholipids. Rat brain also contains a high long-chain fatty acyl-CoA hydrolase activity, however the results clearly demonstrate that long-chain fatty acyl-CoA esters can still be isolated from rat brain. It has been suggested that some sort of control or compartmentalization of the enzyme may exist *in vivo*. However the results from this study indicate that the enzyme is essentially soluble in nature and that it is not localized in any particular cellular compartment. The most likely mechanism for control of the enzyme *in vivo* may be through the binding of the long-chain acyl-CoA esters to a protein. The binding of the thioesters in this way would ensure that the concentration of free or unbound acyl-CoA esters *in vivo* is very low, therefore there would seem to be no role for such an intense hydrolase activity in brain. Long-chain fatty acyl-CoA esters are known to inhibit a number of enzymes, including some involved in fatty acid biosynthesis,eg. fatty acid synthetase (Robinson *et al.*, 1963) and acetyl-CoA carboxylase (Goodridge, 1972). The regulatory significance of fatty acyl-CoA esters as negative effectors has been questioned largely because of the possible non-specific detergent effects of these compounds (Dorsey and Porter, 1968), however Kuo-Hom Lee Hsu and Powell (1975) have suggested that inhibition of citrate synthase by oleoyl-CoA

could not be due solely to the detergent effect of the thioester. The long-chain fatty acyl-CoA hydrolase activity of brain would maintain the concentration of free acyl-CoA esters at a very low level and would therefore ensure that their detergent effect was kept to a minimum. Dils *et al.* (1976) have proposed a role for the long-chain fatty acyl-CoA hydrolase of mammary gland. These workers suggest that the true substrates for the enzyme *in vivo* are the acyl-acyl carrier proteins, which are an integral part of the fatty acid synthetase complex. They propose that the hydrolase acts directly on the synthetase, terminating fatty acid biosynthesis and releasing free fatty acid. A similar role for the hydrolase may exist in brain and the substrate specificity of the enzyme, which has been demonstrated using acyl-CoA esters, would ensure that only fatty acids with chain lengths of C14 and C16 are released. Both of the roles proposed for the long-chain fatty acyl-CoA hydrolase of rat brain release unesterified fatty acids and it is suggested that this enzyme may be essential for the maintenance of the unesterified fatty acid pool of rat cerebral cortex *in vivo*. The long-chain fatty acyl-CoA hydrolase activity of rat cerebral cortex was not directly affected by neurotransmitters. However if control of the enzyme in the intact cell is mediated through cyclic nucleotides as suggested previously, it is unlikely that any effect would have been observed in the cell-free supernatant. The rapid incorporation of labelled unesterified fatty acids into synaptic membranes indicates that the pool of unesterified fatty acids is in equilibrium with the fatty acids released by the phospholipase

A2-acylase system. Therefore factors that affect the composition of the unesterified fatty acid pool, such as the long-chain fatty acyl-CoA hydrolase, could regulate the fatty acid composition of the membrane phospholipids. However it seems more likely that the composition of the membrane phospholipids is controlled by specific acyltransferases and not solely by the composition of the unesterified fatty acid pool. The precise role of the long-chain fatty acyl-CoA hydrolase must await further investigation.

SUGGESTIONS FOR FURTHER WORK

In the previous section it has been suggested that the deacylation and reacylation of membrane phospholipids may be involved in controlling post-synaptic membrane permeability. At present there is little information on the speed of these enzymic reactions and therefore any direct involvement in the primary events of membrane depolarisation or hyperpolarisation can only be speculative. In addition it is still unclear what proportion of the membrane phospholipids are involved in this sequence of events. It would be of interest to know the specificity of the phospholipase A1 and A2 enzymes in brain and whether or not more than one form of these enzymes is present in synaptic membranes. Obtaining information on the phospholipid composition of purified synaptic membranes would be a comparatively simple procedure and this information together with studies on phospholipase specificity may indicate whether the deacylation - reacylation of membrane phospholipids

involves discrete areas of the post-synaptic membrane or the whole membrane itself.

The determination of the kinetic parameters of the deacylation and reacylation reactions poses many problems. Both enzyme systems appear to be membrane bound and the precise nature of their active sites is unclear. Techniques are available for solubilizing membrane-bound enzymes and studying them in an aqueous environment. However such techniques usually alter the kinetic parameters of the enzymes and may then not reflect the true activity of the enzyme when it is attached to the membrane. The assay systems available to date for measuring the deacylation and reacylation reactions are invariably discontinuous and are unsuitable for measuring very fast enzyme reactions. The question of the substrate specificities of membrane-bound phospholipases and the speed of the deacylation and reacylation reactions must await the development of a continuous assay system which could be used to measure the activity of these synaptic membrane-bound enzymes.

An alternative method for estimating the specificity of the deacylation and reacylation of synaptic membrane phospholipids would be to measure the turnover of the fatty acyl groups of individual phospholipids classes, *in vivo*. However the incorporation of labelled acetate into the unesterified fatty acids associated with synaptic membranes, reported in this work, suggests that fatty acids are released from synaptic membrane phospholipids during the

isolation procedure. Therefore an accurate measurement of turnover would be difficult unless the deacylation reaction could be inhibited during the isolation of the synaptic membranes. Measuring the turnover of the phospholipid classes from whole brain may overcome this problem to some degree but would be unlikely to give an accurate measurement of the turnover of individual phospholipid classes within synaptic membranes.

Much of the evidence discussed in this thesis suggest that the neurotransmitter stimulated turnover of phosphatidylinositol is primarily a pre-synaptic occurrence. However there is also some evidence to suggest that acetylcholine stimulated phosphatidylinositol breakdown and resynthesis may also occur in the post-synaptic membrane and that the phosphatidylinositol response may be mediated via a muscarinic acetylcholine receptor. Furthermore phosphatidylinositol is associated with a proteolipid isolated from brain by Lunt *et al.*, which is believed to be an acetylcholine receptor (see Michell, 1975 for references and further discussion). These workers have shown that the phosphorylinositol group undergoes stimulated turnover. The stimulated turnover of phosphatidylinositol and its association with a receptor may be indicative of a specific role for this phospholipid in receptor function. Techniques are now available for solubilizing receptor material into aqueous solution, but these have not been applied so far with any success to the muscarinic acetylcholine receptor from brain.

The phospholipase C activity of such a soluble receptor preparation would be an obvious candidate for study and it would also be useful to determine whether or not the diglyceride released by phospholipase C action is hydrolysed further by di- and mono-glyceride lipase action. It would also be of interest to discover whether the isolated receptor shows any deacylation and reacylation activity.

The long-chain fatty acyl-CoA hydrolase activity of rat cerebral cortex was not affected by neurotransmitters. It has been suggested that control by neurotransmitters could be mediated via cyclic nucleotides. Measuring the effectof cyclic nucleotides on the soluble enzyme preparation would not be expected to present any serious experimental problems. However to reproduce any effect in the intact synaptosome using neurotransmitters may be more difficult. The assay systems available for measuring the hydrolase activity have been described in Methods 12 . Both assays involve measuring changes in optical density; following such changes in a synaptosome suspension poses many problems, of which the major one may be the permeability of the plasma membrane to reagents and substrates. Hydrolase activity could also be assayed using radioactively labelled long-chain fatty acyl-CoA esters and measuring the released radioactively labelled fatty acid, however such an assay system would again depend upon the permeability of the plasma membrane to the radioactively labelled substrate. Some preliminary experiments, not reported in the main part of theis thesis, have indicated that either the reagent (DTNB) or the acyl-CoA ester substrate may not freely

permeate the synaptosome membrane, however it must be emphasised that these experiments are only preliminary and are by no means conclusive.

It is hoped that further experimentation on brain lipid metabolism will be useful in aiding our understanding of the molecular events which govern the changes in membrane permeability taking place during the process of synaptic transmission.

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PUBLISHED WORK

The Incorporation of [1 - ^{14}C] Acetate into unesterified Fatty Acids in Rat Cerebral Cortex *In Vivo*.

R.W. Bonser & G.G. Lunt

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The Long-Chain Fatty Acyl-CoA Hydrolase Activity of Rat Cerebral Cortex.

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APPENDIX

The data for Fig. 5 is presented below in Table 13.

TABLE 13. THE INCORPORATION OF [1 - ^{14}C] ACETATE INTO THE UNESTERIFIED FATTY ACIDS OF RAT CEREBRAL CORTEX *IN VIVO*

Expt.	INCORPORATION TIME (h)	SPECIFIC RADIOACTIVITY (d.p.m./mg methyl ester)	MEAN ± S.E.
1	0.5	28354	
2	0.5	33961	36901±5974
3	0.5	48388	
1	1.0	55101	
2	1.0	74507	58714±8283
3	1.0	46537	
1	2.0	8652	
2	2.0	14372	12370±1863
3	2.0	14086	
1	3.0	11280	
2	3.0	9759	10270±505
3	3.0	9772	
1	6.0	6004	
2	6.0	10413	8145±1275
3	6.0	8019	
1	12	10047	10163±116
3	12	10279	
1	24	14910	
2	24	29872	22526±4326
3	24	22797	
1	5 days	11580	
2	5 days	10091	9554±1353
3	5 days	6991	

Table 14 continued

EXPT.	INCORPORATION TIME (min)	SPECIFIC RADIOACTIVITY (d.p.m./mg methyl ester)	MEAN ± S.E.
1	30	7396	
2	30	9390	
3	30	9844	9277 ± 666
4	30	10480	
1	60	14148	
2	60	12119	13160±1125
3	60	15749	
4	60	10625	
2	90	16989	
3	90	11488	13482±1523
4	90	11969	

TABLE 15. INCORPORATION OF [1 -^{14} C] ACETATE INTO THE FATTY ACIDS OF NEUTRAL GLYCERIDES FROM RAT CEREBRAL CORTEX *IN VIVO*

EXPT.	INCORPORATION TIME (min)	SPECIFIC RADIOACTIVITY (d.p.m./mg methyl ester)	MEAN ± S.E.
1	0.0	707	
2	0.0	1083	958±86
3	0.0	989	
4	0.0	1055	
1	1.0	3573	
2	1.0	2671	3005±369
3	1.0	3657	
4	1.0	2121	
1	2.5	3409	
2	2.5	4947	4204±323
3	2.5	4419	
4	2.5	4034	
1	5.0	7132	
2	5.0	7998	7518±395
3	5.0	6606	
4	5.0	8335	
2	10	12754	
3	10	13539	13848± 738
4	10	15252	
1	20	13761	
3	20	12824	12989± 406
4	20	12383	
1	30	10962	
2	30	15027	
3	30	11336	11858±1292
4	30	16274	
5	30	8980	
6	30	8573	
1	60	9051	
2	60	11690	8963±1600
4	60	6148	
1	90	11137	
2	90	11844	10252±779
3	90	9683	
4	90	8345	

TABLE 16. INCORPORATION OF [1 - ^{14}C] ACETATE INTO THE FATTY ACIDS OF PHOSPHOLIPIDS FROM RAT CEREBRAL CORTEX *IN VIVO*

EXPT.	INCORPORATION TIME (min)	SPECIFIC RADIOACTIVITY (d.p.m./mg methyl ester)	MEAN ± S.E.
1	0.0	504	
2	0.0	632	595±30
3	0.0	635	
4	0.0	610	
1	1.0	1935	
2	1.0	1198	1499±194
3	1.0	1718	
4	1.0	1146	
1	2.5	1760	
2	2.5	2135	1997±81
3	2.5	2059	
4	2.5	2037	
1	5.0	2077	
2	5.0	2316	2373±108
3	5.0	2621	
4	5.0	2480	
1	10	1190	
3	10	1799	1627±264
4	10	1973	
1	20	2085	
3	20	2749	2197±292
4	20	1756	
1	30	2095	
2	30	1752	
3	30	4182	2759±456
4	30	2050	
5	30	4174	
6	30	2305	
1	60	2431	
2	60	2787	2759±423
3	60	3910	
4	60	1909	
1	90	3191	
2	90	3581	3009±232
3	90	2632	
4	90	2630	

The data for Fig. 8 is presented below in Table 17.

TABLE 17. INCORPORATION OF [1 - ^{14}C] ACETATE INTO THE LONG-CHAIN FATTY ACYL-CoA ESTERS OF RAT BRAIN *IN VIVO*

INCORPORATION TIME (min)	YIELD OF ACYL-CoA ESTER(nmol)	TOTAL RADIOACTIVITY (d.p.m.)	SPECIFIC RADIOACTIVITY (d.p.m./nmol acyl-CoA ester)
0.0	2.97	3790	1276
0.0	4.40	6038	1372
0.0	4.73	6926	1445
0.25	1.58	2960	1882
0.25	1.45	3042	2095
0.25	1.69	3377	1993
0.5	2.30	3278	1425
0.5	3.15	3492	1109
0.5	2.18	3343	1533
1.0	3.58	1642	462
1.0	2.97	2051	691
1.0	2.75	2058	748
3.0	1.98	1204	608
3.0	3.52	1333	379
3.0	2.97	1538	518
3.0	4.40	1333	303
5.0	2.70	922	342
5.0	5.28	2532	480
5.0	3.96	2437	615
10	3.82	2350	615
10	2.40	1839	766
10	2.10	1314	626

Torda (1974) proposed a model of a depolarization–hyperpolarization cycle, in which she suggested that the regulatory subunit of triphosphoinositide phosphomonoesterase constitutes a part of the post-synaptic nicotinic acetylcholine receptor.

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The Long-Chain Fatty Acyl-Coenzyme A Hydrolase Activity of Rat Cerebral Cortex

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Unesterified fatty acids constitute a small but very labile pool of the brain lipids (Rowe, 1964; Lunt & Rowe, 1968, 1969, 1971; Bazan & Joel, 1968; Bazan, 1971). Electroshock and certain convulsive drugs increase the concentration of unesterified fatty acids in brain (Bazan, 1970, 1971), and it has been suggested that they may be involved in the changes in membrane permeability that accompany synaptic transmission (Lunt & Rowe, 1971; Vyvoda & Rowe, 1973). The source of the acids is not yet known. Vyvoda and Rowe (1972) showed that guinea-pig brain contained mono-, di- and triglyceride lipase activities, whose action on exogenous substrates is modulated by neurotransmitters, and Gullis & Rowe (1975*a*) have reported the presence of a phospholipase A2 in guinea-pig synaptic membranes, which is also sensitive to neurotransmitters. It is probable that, under conditions of increased transmitter release *in vitro* or *in vivo*, the hydrolysis of neutral glycerides and phospholipids contributes markedly to the unesterified fatty acid pool. We have previously shown, however, that, under non-stimulated conditions in rat cerebral cortex *in vivo*, fatty acids appear to be incorporated into neutral glycerides and phospholipids, rather than being derived from them (Bonser & Lunt, 1976). We suggested that an alternative source of the unesterified fatty acids may be a long-chain fatty acyl-CoA hydrolase activity. The presence of such an activity (palmitoyl-CoA hydrolase; EC 3.1.2.2) in brain has been known for some time (Srere *et al.*, 1958; Vignais & Zabin, 1958). If, as suggested previously (Bonser & Lunt, 1976), a long-chain fatty acyl-CoA hydrolase activity contributes to the maintenance of the brain pool of unesterified fatty acids it may be that the enzyme activity is sensitive to neurotransmitters or to cyclic nucleotides, as is the case of the phospholipase A2 described by Gullis & Rowe (1975*a*,*b*).

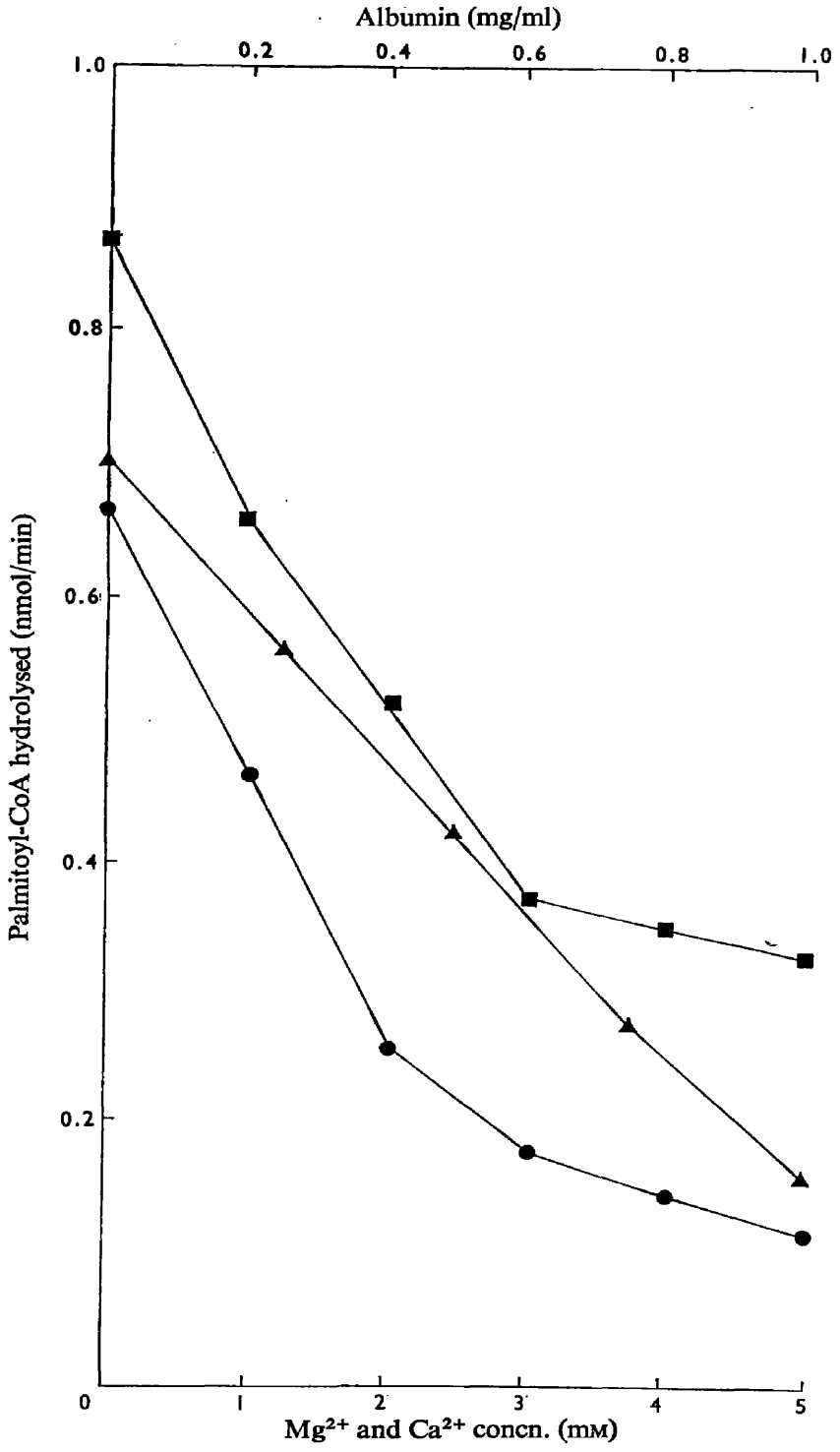

Fig. 1. *Effects of Ca^{2+}, Mg^{2+} and albumin on the activity of the long-chain fatty acyl-CoA hydrolase of rat cerebral cortex*

The activity was measured in a 17500$g_{av.}$ supernatant fraction of rat cerebral cortex. ■, Mg^{2+}; ●, Ca^{2+}; ▲, albumin.

The concentration of long-chain fatty acyl-CoA esters in rat brain was found to be 12.5±0.42nmol/g wet wt. (mean± S.E.M., 14 determinations). The long-chain fatty acyl-CoA hydrolase activity was measured in a 17500$g_{av.}$ supernatant fraction of rat cerebral

Table 1. *Subcellular distribution of long-chain acyl-CoA hydrolase activity in rat cerebral cortex*

Subcellular fractions were prepared from whole cortex as described by Lapetina *et al.* (1967). One enzyme unit is defined as the activity required to hydrolyse 1 μmol of palmitoyl-CoA/min.

		Enzyme (units)		Enzyme (percentage of total units)	
Fraction	Isolation conditions	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Total homogenate	10% Homogenate in 0.32M-sucrose	32.73	35.08	100	100
Nuclear pellet	900*g*, 10min, 2 washings	6.49	6.12	19.8	17.4
Crude mitochondrial pellet	10000*g*, 20min, 1 washing	3.14	4.18	9.5	11.9
10000*g* Supernatant	10000*g*, 20min	25.75	26.59	78.6	75.7
Overall recovery		35.38	36.89	108	105

cortex prepared as described by Kurooka *et al.* (1972). The enzyme exhibited a broad pH profile with maximal activity between pH 8.0 and 8.5. The K_m of the enzyme for palmitoyl-CoA and oleoyl-CoA was 1.4×10^{-6}M in each case. The results in Fig. 1 demonstrate that the long-chain fatty acyl-CoA hydrolase is inhibited both by Mg^{2+} and Ca^{2+}. Bivalent cations probably complex with the pyrophosphate group on the CoA molecule, producing a compound that is a poor substrate for the enzyme. Fig. 1 shows that the enzyme is also inhibited by bovine serum albumin. Inhibition is probably due to binding of the acyl-CoA substrate, since the inhibition can be reversed by adding excess of substrate. Long-chain fatty acyl-CoA hydrolase activity remained unaffected by adrenaline, noradrenaline, 5-hydroxytryptamine, acetylcholine and carbachol at concentrations ranging from 10 to 100μM. The hydrolysis of palmitoyl-CoA by the enzyme was not affected by the addition of palmitic acid at concentrations up to 100μM.

Table 1 shows the subcellular localization of long-chain fatty acyl-CoA hydrolase. Between 75 and 80% of the total enzyme activity can be recovered in the $10000g_{av.}$ supernatant. Approx. 10% of the activity is associated with the crude mitochondrial fraction. The crude mitochondrial pellet from the $10000g_{av.}$ centrifugation was osmotically shocked by homogenizing it in distilled water, as described by Lapetina *et al.* (1967). The shocked pellet was then centrifuged at $100000g_{av.}$ for 1h. Approx. 75% of the enzyme activity associated with the mitochondrial fraction was recovered in the $100000g_{av.}$ supernatant. These results indicate the enzyme is soluble.

Brain has a higher long-chain fatty acyl-CoA hydrolase activity than other tissues (Kurooka *et al.*, 1972). It is still possible, however, to isolate long-chain fatty acyl-CoA esters, suggesting some sort of control or compartmentalization of the long-chain fatty acyl-CoA hydrolase *in vivo*. The concentration of unesterified fatty acid in rat cerebral cortex is approximately 0.96μmol/g wet wt. (Bonser & Lunt, 1976), and hydrolysis of long-chain fatty acyl-CoA esters *post mortem* could only account for approx. 1.3% of this yield. A rapidly turning-over pool of acyl-CoA esters coupled with the hydrolase activity could, however, make a significant contribution to the unesterified fatty acid pool. The soluble enzyme preparation used in these studies showed no stimulation by neurotransmitters, but the possibility remains that control of the enzyme may be achieved in the intact cell via a cyclic nucleotide system such as has been reported by Gullis & Rowe (1975*a*,*b*) for the phospholipase A2 activity of guinea-pig brain.

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Proteolipid-Bound Unesterified Fatty Acids in Rat Cerebral Cortex: Incorporation of [1-^{14}C]Acetate *in vivo*

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It is known that the total brain pool of unesterified fatty acids is highly labile and metabolically active (Rowe, 1964; Lunt & Rowe, 1968, 1971; Bonser & Lunt, 1976), but no information is available about the metabolism of the proteolipid-bound unesterified fatty acids. In view of the possible role of proteolipids as synaptic receptors [see De Robertis (1975) for review] and the probable involvement of unesterified fatty acids in synaptic-membrane function (Lunt & Rowe, 1971; Vyvoda & Rowe, 1973; Gullis & Rowe, 1975*a,b,c*) we have investigated the incorporation of radioactively labelled acetate into the proteolipid-bound unesterified fatty acid pool.

Total lipid extracts were prepared by homogenizing excised cerebral cortex in 20vol. of chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). The washed total lipid extract was mixed with 4vol. of diethyl ether at −20°C to precipitate total proteolipids (Soto *et al.*, 1969). The proteolipids were further fractionated by chromatography on columns (20cm×2cm) of Sephadex LH20 equilibrated in chloroform (Soto *et al.*, 1969). Samples of total proteolipids and of proteolipid peaks from the Sephadex LH 20 columns were analysed for unesterified fatty acid by two procedures. Procedure 1 involved treating the proteolipid preparation directly with diazomethane; the resultant methyl esters of fatty acids were then extracted in light petroleum. In procedure 2, the proteolipid preparations were extracted with acetone; the acetone extract was then methylated with diazomethane, and the methyl esters of fatty acids were collected as before. A detailed account of this method and of the subsequent determination of the methyl esters by g.l.c. has been given (Lunt & James, 1976), and these procedures were used without modification in the experiments described here.

Groups of 12 rats received 2μCi of [1-^{14}C]acetate by intraventricular injection. After various time-intervals (2min, 10min, 20min, 1h, 6h, 12h, 24h) the animals were killed, and the unesterified fatty acids of the total diethyl ether-precipitated proteolipids measured. Significant incorporation of radioactivity was seen only after 12–24h incorporation periods; consequently in all subsequent experiments a 24h incorporation period was used. The distribution of radioactivity between the unesterified fatty acids of the various lipid fractions is shown in Table 1 and it can be seen that there is metabolic heterogeneity within the proteolipid-bound pool of unesterified fatty acids.

The total proteolipid fractions from animals that had received an intraventricular injection of [1-^{14}C]acetate were fractionated by column chromatography on Sephadex LH 20. The elution pattern of proteolipid protein was consistent with that obtained previously (Lunt & James, 1976). The four main proteolipid peaks were collected, and

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THE INCORPORATION OF [1-^{14}C]ACETATE INTO UNESTERIFIED FATTY ACIDS IN RAT CEREBRAL CORTEX *IN VIVO*

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(*Received* 22 *May* 1975. *Accepted* 8 *July* 1975)

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THE FIRST detailed report of the presence of unesterified fatty acids (UFA) in rat brain was made by ROWE (1964). Subsequent work has confirmed that UFA constitute a small, but highly labile, pool of the brain lipids (LUNT & ROWE, 1968, 1969, 1971; BAZAN & JOEL, 1968). Marked increases in the levels of the acids are seen under conditions of intense synaptic activity such as occurs during electro-shock treatment (BAZAN, 1971).

The sources of UFA in the brain have not yet been defined. Phospholipase activity has been proposed as a contributory factor (BAZAN, 1970) and triglyceride breakdown may also add to the UFA pool but cannot alone account for the observed increases (ROWE, 1969; LUNT & ROWE, 1971).

It has been shown previously that there is rapid incorporation of ^{14}C-labelled acetate into the UFA of cerebral cortex *in vitro* (ROWE, 1964; LUNT & ROWE, 1968). More recently the rapid incorporation of ^{14}C-labelled UFA into neutral glycerides and into phospholipids of cerebral cortex *in vivo* has been reported (SUN & HORROCKS, 1971; YAU & SUN, 1973; 1974).

In the experiments presented here the incorporation of [1-^{14}C]acetate into UFA and into the fatty acids of neutral glycerides and of phospholipids has been measured *in vivo*.

MATERIALS AND METHODS

Animals

Adult (3–4 months, 150–200 g) male Wistar rats (Animal Suppliers, Welwyn) were used throughout the work.

Radioactive precursors

Na-[1-^{14}C]acetate (1 mCi; 59 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, as was [1-^{14}C]palmitic acid (50 μCi; 59 mCi/mmol) and glycerol tri-[1-^{14}C]palmitate (250 μCi; 46 mCi/mmol).

The [1-^{14}C]acetate was dissolved in distilled water to give a final activity of 0·1 mCi/ml; [1-^{14}C]palmitic acid was dissolved in diethyl ether to give a final activity of 0·5 μCi/ml; glycerol tri-[1-^{14}C]palmitate was dissolved in diethyl ether to give a final activity of 2·5 μCi/ml.

All radioactive measurements were made in a Packard Model 3385 liquid scintillation spectrometer using NE 260 scintillant (Nuclear Enterprises, Reading). Samples were counted for 10 min or 10,000 counts and corrected for counting efficiency and background.

Other materials

General reagents and solvents were from Fisons Ltd. (Loughborough) or B.D.H. Ltd., (Poole). All solvents were redistilled before use.

Radioactive labelling of fatty acids in vivo

Animals received 20 μl (2 μCi) of the aqueous solution of [1-^{14}C]acetate by injection into the right lateral ventricle (NOBLE *et al.*, 1967). The rats were decapitated at varying time intervals after injection and the brains rapidly removed. The cerebral cortex was substantially freed from white matter by dissection and the cortical tissue homogenised for 5 min in 5 ml ice-cold 0·14 M-barbital–acetate buffer, pH 7·4 (MICHAELIS, 1931). A further 20 ml ice-cold buffer were added to the homogenate and the tissue precipitated by centrifugation at 60,000 × g_{av} for 10 min. The tissue was washed twice by resuspending in 20 ml of buffer and sedimented by centrifugation. The temperature was maintained at 0–4°C throughout the washing procedure.

Extraction and separation of lipid classes

The washed tissue pellet was extracted with 20 vol of chloroform:methanol (2:1, v/v) (FOLCH-PI *et al.*, 1957). The total lipid extract was washed once with 0·2 vol of 0·1 M-sodium acetate and twice with 'theoretical upper phase' (FOLCH-PI *et al.*, 1957) containing 0·5 M-sodium acetate (CLAYTON & ROWE, 1966).

The washed lipid extract was evaporated to dryness under reduced pressure and the dry lipid film was extracted with 3 × 5 ml acetone to remove neutral lipids. The neutral lipid extract was evaporated to dryness and redissolved in 10 ml water–ethanol (1:1, v/v) and the neutral glycerides were separated from the UFA using the solvent extraction

Abbreviations used: UFA, unesterified fatty acids.

TABLE 1. RECOVERY OF [1-^{14}C]ACETATE IN FATTY ACID FRACTIONS FROM RAT CEREBRAL CORTEX LIPIDS

Fraction	Total radioactivity of fraction (d.p.m.)	% of radioactivity recovered after injection attributable to unmetabolised acetate*
Initial tissue homogenate	$4{\cdot}4 \times 10^6$	—
UFA	254 ± 19 (5)	$7{\cdot}5 \pm 1{\cdot}1$ (4)
Neutral glyceride fatty acids	155 ± 5 (3)	$0{\cdot}9 \pm 0{\cdot}1$ (4)
Phospholipid fatty acids	110 ± 14 (3)	$3{\cdot}6 \pm 0{\cdot}4$ (4)

2 μCi of [1-^{14}C]acetate were added to an homogenate of the cerebral cortex in ice-cold 0·14 M-acetate–barbital buffer. The tissue was washed and the fatty acids isolated as described in Methods; the radioactivity of the fractions was measured. *These values are calculated from the data in Fig. 1 using the levels of incorporation seen at 0 min. which represents a true incorporation period of 2·0 min. All values are given as mean ± S.E. (no. of experiments).

procedure described by ROWE (1964). Neutral glycerides and the phospholipid from the acetone-extraction procedure were hydrolysed by the method of LOVELOCK *et al.*, (1959). The UFA and the fatty acids from phospholipids and neutral glycerides were converted to their methyl esters by treatment with freshly prepared diazomethane (JAMES, 1960).

Other estimations

The methyl esters of fatty acids were measured by gas chromatography using a Pye Series 104 gas chromatogram fitted with a flame ionization detector as described previously (LUNT & JAMES, 1976).

Proteins were estimated by the method of LOWRY *et al.* (1951).

RESULTS

Washing procedures and control experiments

Preliminary experiments showed that at short incorporation times a considerable amount of unmetabolised [1-^{14}C]acetate is present in the excised cerebral cortex which leads to contamination of the isolated fatty acid fractions. Consequently a series of washing procedures was devised and is described in the Methods section. A series of control experiments was done to establish the degree of contamination of the lipid fractions with [1-^{14}C]acetate. Cerebral cortices were removed from non-injected rats and homogenised in ice-cold 0·14 M-acetate–barbital buffer. Sodium acetate, (20 μl; 2 μCi) was then added to the homogenate which was subjected to the washing and lipid extraction procedures described in Methods. The amount of radioactivity recovered in the fatty acid fractions is shown in Table 1.

It was found that no further decreases in the levels of radioactivity of the fatty acid fractions were obtained by increasing the number of washes of either the tissue homogenate or of the lipid extracts.

Recovery of UFA and neutral glycerides

Experiments were done to establish the losses of UFA and of neutral glycerides incurred in the washing procedures. [1-^{14}C]palmitic acid (100 μl; 0·25 μCi) and glycerol tri-[1-^{14}C]palmitate (100 μl; 0·25 μCi) were added to homogenates of cerebral cortex from individual rats. After the three washes with 0·14 M-barbital–acetate buffer 81% and 85% of the initial palmitic acid and tripalmitin respectively were recovered in the tissue pellet. After the lipid extraction and three washes of the total lipid extract with theoretical upper phase 77% and 80% of the initial palmitic acid and tripalmitin respectively were retained in the washed lipid extract. The yield of UFA was 9·6 ± 0·35 μmol/g protein (mean ± S.E. of 64 determinations).

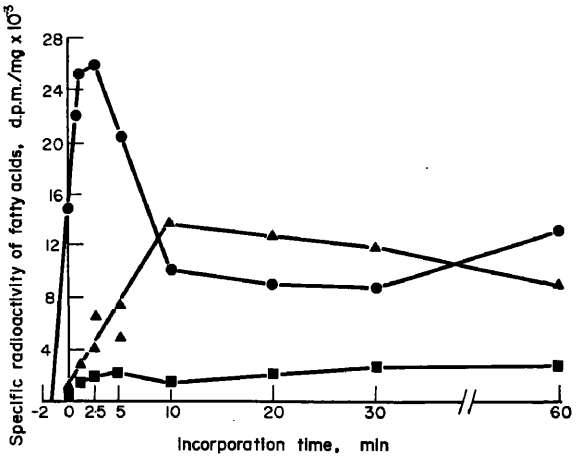

FIG. 1. The incorporation of [1-C^{14}]acetate into the fatty acids of rat cerebral cortex *in vivo*. Rats received 2 μCi of [1-C^{14}]acetate by intraventricular injection. After decapitation the cortex was rapidly removed and fatty acids isolated from the washed cortical homogenate as described in Methods. The incorporation time represents the period from the time of injection to the time of decapitation. ●—UFA: ▲—fatty acids of neutral glycerides: ■—fatty acids of phospholipids. Each point is the mean of 4 separate experiments; variation within each group of 4 results was not more than 10%.

The incorporation of [1-^{14}C]acetate into fatty acids

Rats received 2 μCi (20 μl) of [1-^{14}C]acetate by intraventricular injection and were killed at varying time intervals. UFA and the fatty acids of neutral glycerides and of phospholipids were isolated and their specific radioactivities determined. The time between decapitation and homogenisation of the excised cortex was 2·0 ± 0·25 min (40 determinations). The pattern of incorporation of acetate into the fatty acid fractions is shown in Fig. 1. During the 2 min period between decapitation and homogenisation acetate incorporation could proceed rapidly. Therefore the true 'zero incorporation time' is represented by the −2·0 min value in Fig. 1.

DISCUSSION

Slices of mouse cerebral cortex incubated *in vitro* at 37°C showed a rapid incorporation of [1-^{14}C]acetate into UFA (LUNT & ROWE, 1968). DHOPESHWARKAR *et al.* (1971) measured the incorporation of [1-^{14}C]acetate into the total fatty acids of rat brain lipids *in vivo* and found a very rapid incorporation into palmitic and stearic acids. ROWE (1964) had shown earlier that in rat brain *in vitro* [1-^{14}C]acetate was incorporated primarily into palmitic acid, probably by a chain elongation of myristate rather than by synthesis *de novo* and CAREY (1975) has reported a similar situation in incubated slices of rabbit cerebral cortex. Measurements have also been made of the rates of incorporation of exogenous UFA into both neutral glycerides and phospholipids in brain *in vivo* (SUN & HORROCKS, 1971; YAU & SUN, 1973, 1974). Thus, there is now ample evidence that the UFA of cerebral cortex constitute a metabolically active pool of brain lipids. However the relationship between the three main pools of fatty acids, namely, UFA, and the esterified fatty acids of neutral glycerides and of phospholipids is not clear.

Examination of Table 1 shows that the washing procedures used in our experiments reduce the amount of [1-^{14}C]acetate contamination of the isolated fatty acid fractions to a very low level. Thus we consider the rapidly increasing radioactivity of the fatty acid fractions seen in Fig. 1 to be indicative of metabolic incorporation of acetate. The level of incorporation into UFA seen at 0 min in Fig. 1. is considerably higher than could be accounted for by contamination from unmetabolised [1-^{14}C]acetate (see Table 1) indicating that incorporation proceeds rapidly during the 2 min period between decapitation and homogenisation of the tissue.

The most rapid incorporation is seen in the UFA which have a turnover time of 5–6 min. This value compares favourably with the half-life of approx 5 min for intracerebrally-injected oleic acid and arachidonic acid in mouse brain reported by YAU & SUN (1973, 1974). The time course of the appearance of labelled acetate in the neutral glyceride fraction also agrees well with the incorporation pattern of exogenous palmitic, oleic and stearic acids into di- and triaclgycerols, in mouse brain (SUN & HORROCKS, 1971; YAU & SUN, 1973, 1974).

The source of the brain UFA is not yet known. VYVODA & ROWE (1973) showed that guinea-pig brain contained mono-, di- and triglyceride lipase activities whose action on exogenous substrates is modulated by noradrenaline, 5-hydroxytryptamine and adrenaline. GULLIS & ROWE (1973) showed also that nerve ending membranes from guinea-pig brain contain a phospholipase A_2 which is stimulated by noradrenaline. Thus it is probable that under conditions of increased transmitter release *in vitro* or *in vivo* the hydrolysis of neutral lipids and of phospholipids contributes markedly to the UFA pool. Our results however suggest that under normal conditions UFA are incorporated into neutral glycerides and phospholipids rather than being derived from them and we suggest that lipolysis is not the major source of UFA in a non-stimulated state. An alternative source of free acids may be acyl CoA-deacylase activity and the presence of a palmitoyl-CoA deacylase (palmitoyl-CoA hydrolase EC 3.1.2.2.) in brain has been known for some time. (SRERE *et al.*, 1958; VIGNAIZ & ZABIN, 1958). It is unlikely that the breakdown of acylCoA esters post mortem accounts for the total UFA pool since the total coenzyme A content of rat brain is approx 0·09 μmol/g fresh tissue (KAPLAN & LIPMANN, 1948; JAENICKE & LYNEN, 1960), compared with UFA levels of about 1 μmol/g fresh tissue. However, a deacylase activity could ensure the maintenance of a small, labile pool of UFA *in vivo* and such a system would be consistent with the acetate-labelling pattern reported here in which UFA appear to be precursors to neutral glycerides and phospholipids as seen by SUN & HORROCKS (1971) and YAU & SUN (1973, 1974). The role of UFA is not yet established. The suggestion has been made that they may be involved in the regulation of the changes in synaptic membrane permeability that are an essential part of chemical transmission. (LUNT & ROWE, 1971; VYVODA & ROWE, 1973). It has been reported that in rat cerebral cortex part of the UFA pool is associated with a proteolipid thought to be part of the cholinergic receptor system (LUNT & PETCH, 1974). The lipid extraction procedures used in the present work leads to the dissolution of the proteolipid-UFA complex and these data reflect only the average metabolic activity of the total cortex UFA pool. It is hoped that further studies of the metabolic activity of the individual pools of UFA and of their coenzyme A esters may assist in the elucidation of their possible role in synaptic transmission.

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